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THE UNIVERSITY OF ALBERTA

ISOLATION, CHARACTERIZATION AND NUCLEOTIDE SEQUENCE OF SIX HUMAN TYROSINE ACCEPTING tRNA GENES

© BY

JAMES M. MacPHERSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY.

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

FALL, 1988

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1

ABSTRACT

Three fragments of human DNA isolated from a human λ-Charon-4A recombinant DNA library were found to contain at least six human tRNA genes. Two of these recombinants were suggested, on the basis of homology to a tRNA^{Tyr} gene probe, to be single solitary genes while the other four tRNA genes are contained within a 9.2-kb length of DNA. Nucleotide sequence analysis of short regions from two bacteriophage clones, representing five of the six genes, revealed that all were human tRNA^{Tyr} genes and that each gene was interrupted by an intron. These were the first examples of human (and mammalian) tRNA genes to contain intervening sequences. The introns varied both in nucleotide sequence and length. There was little evidence of homology between them. The gene-coding regions were identical in sequence, with the exception of G/A polymorphisms at two positions within the 3'-half of these genes. Extensive regions of 5'-flanking sequence homology have been identified, as well as some limited 3'-flanking sequence homology. Experiments using human *in vitro* transcription systems revealed that these genes are expressed but at very different levels of efficiency.

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LIST OF ABBREVIATIONS

AP	ampicillin
bp	base pair(s)
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dNTP	deoxynucleoside 5'- triphosphate
dTTP	deoxythymidine 5'-triphosphate
ICR	internal control region
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase(s) or 1000bp
mRNA	messenger RNA
nt	nucleotide(s)
PAGE	polyacrylamide gel electrophoresis
pfu/ml	plaque forming units/ml
Ψ	pseudouridine
rf	replicative form
rRNA	ribosomal RNA
RRN	ribosomal RNA genes
TC	tetracycline
TCA	trichloroacetic acid
tDNA	DNA complementary to tRNA
TFIIIB	transcription factor IIIB
TFIIIC	transcription factor IIIC
tRNA	transfer RNA

X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION

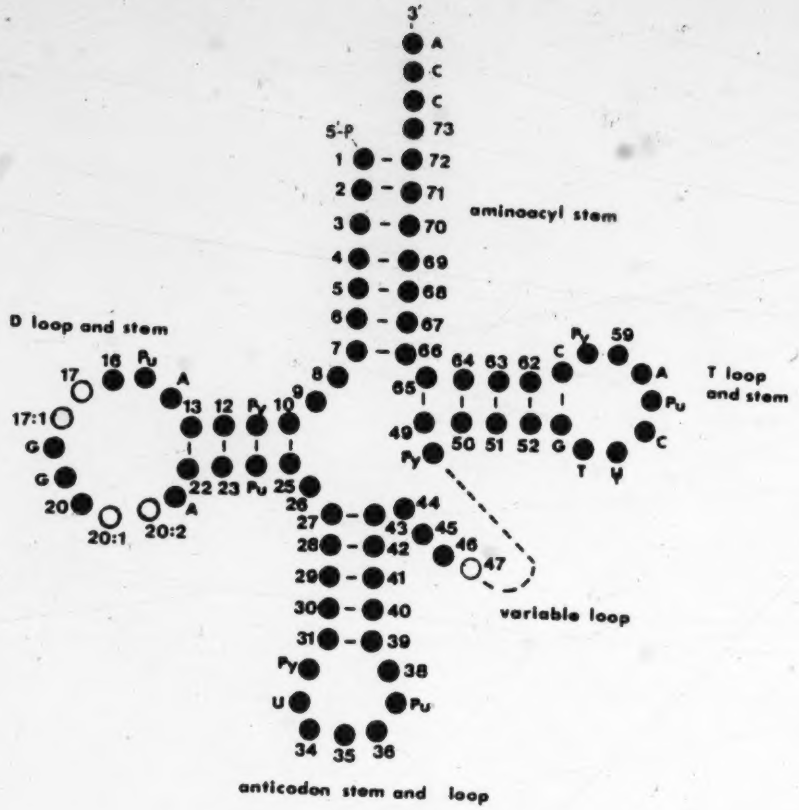
(a) The structure of transfer RNA

The first nucleotide sequence of an RNA molecule was determined by Holley *et al.* (1965). This molecule was an alanine accepting tRNA ($tRNA^{Ala}$) from yeast. The most recent compilation of sequences includes 413 mature tRNA sequences and 665 genes for both bacterial and eukaryotic tRNAs (Sprinzl *et al.,* 1987). The study of tRNA has led to an increased understanding of the smallest known biologically active nucleic acids. The major function of tRNA is its role in translation, where tRNA is essential for initiation of protein synthesis and elongation of the growing polypeptide chains. During protein synthesis, the amino acid sequence of the polypeptide is determined by the interaction of the tRNA and the mRNA on the ribosome. The tRNA structure must, therefore, contain considerable information to ensure the necessary interactions with the proteins and other RNA molecules involved in translation.

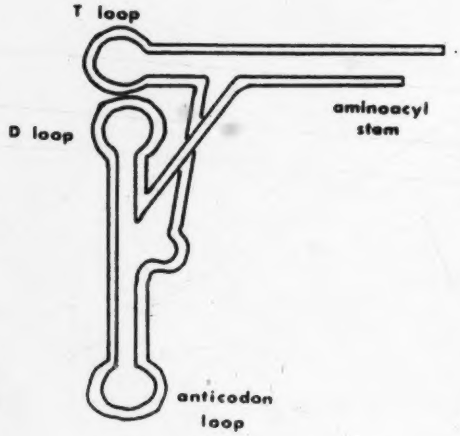
Holley and his colleagues (*op. cit.*) were the first to propose that $tRNA^{Ala}$ from yeast had a unique secondary structure, the model of which was based upon Watson and Crick base pairing interactions between separate regions of the tRNA molecule. Since then, the now-familiar cloverleaf structure (one of the three possibilities suggested; *op cit.*) has been used to depict the secondary structure of tRNAs. The Watson and Crick base pairing format results in three stem-loop regions which are common to most functional tRNA molecules (Fig. 1). The major features of this model are the stem-loop structures and the presence of a 5'-phosphate and the 3'-

Figure 1. The structure of tRNA. (a) The secondary structure of tRNA. The dark circles represent nucleotides which are always present. Open circles denote nucleotides which are not present in every tRNA. For invariant bases, the actual base is indicated. Semi-invariant bases are represented by Py (pyrimidine) or Pu (purine). (b) The schematic tertiary structure of tRNA. The molecule shown in (a) can fold into a small L-shaped structure with the amino acid-accepting stem at one end and the anticodon at the other. (c) The three dimensional configuration of a mature tRNA. (Adapted from Sprinzl *et al.*, 1987)

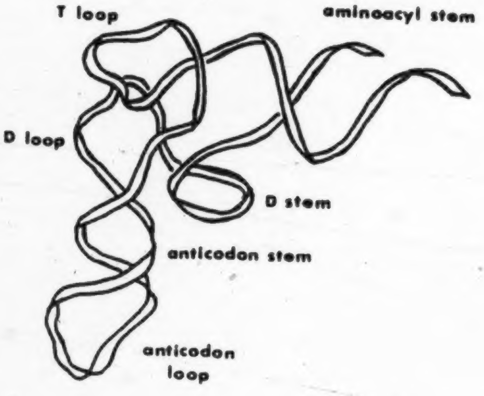
a


A
C
C
5'-P
aminoacyl stem
D loop and stem
Pu
A
17:1
Py
G
G
20:1
20:2
A
Pu
C
Py
A
Pu
C
T loop and stem
G
T
Ψ
Py
variable loop
Py
U
Pu
anticodon stem and loop

b


T loop
D loop
aminoacyl stem
anticodon loop

c


T loop
aminoacyl stem
D loop
D stem
anticodon stem
anticodon loop

terminal-CCA trinucleotide sequence to which the amino acid is esterified. The aminoacyl stem consists of seven base pairs, the D-stem has three or four, and the anticodon-stem and T-stem each contain five base pairs. The anticodon-loop and T-loop each contain seven nonpaired nucleotides, while the length of the variable-loop is dependent upon the specific tRNA. The D-loop has between seven and ten unpaired nucleotides, also depending upon the particular tRNA. All tRNAs have invariant and semi-invariant nucleotides which are involved in the complex L-shaped tertiary structure of mature tRNA (Clark, 1978).

The cloverleaf configuration by which the secondary structure of tRNAs is often depicted is rather misleading as the molecules, when in the functional L-shaped form, are very compact. The double stranded regions of the tRNAs are present in the tertiary configuration, but their arrangement in space creates two helices at right angles to each other. The region of the bend, between the two double stranded regions, contains the D and T loops. Thus, the amino acids are esterified at one end of the molecules while the anticodons are at the other end of the tRNAs. These tertiary structures are created and maintained by hydrogen bonds between bases which are unpaired in the secondary structure of the tRNAs. Most of the invariant and semi-invariant bases are important in the formation of the tertiary structure hydrogen bonds (Clark, 1978).

The coding regions of tRNA genes vary in length from 72 to 90 bp and contain certain common structural features. Each gene must code for the stem-loop elements and several other invariant and semi-invariant bases which contribute to the mature tRNA structure.

(b) Alternative functions of tRNAs

Transfer RNAs engage in activities other than protein synthesis. Soffer (1980) reported that certain charged tRNAs, for example, those for lysine, alanine and arginine, can donate their amino acids to modify some proteins post transcriptionaly. Transcription of the histidine operon of *Salmonella typhimurium* depends upon the cellular concentration of charged $tRNA^{His}$. As the level of charged $tRNA^{His}$ becomes depleted, the histidine operon becomes increasingly expressed (Lewis and Ames, 1972). Transfer RNA has been shown to regulate the transport of leucine, isoleucine and valine into *E. coli* (Quay and Oxender, 1980). More recent evidence suggests that one *E. coli* tRNA may be involved in the maintenance of DNA replication and that the *E. coli* $tRNA_1{}^{Ser}$ gene is required for cell division (Mullin *et al.,* 1984; Tamura *et al.,* 1984).

Eukaryotic systems also have a variety of specialized functions for tRNA. A $tRNA^{Trp}$ functions as the primer for reverse transcription of avian sarcoma virus, and a $tRNA^{Pro}$ is responsible for priming the replication of murine leukemia virus (Dahlberg, 1980). Other functions for eukaryotic tRNAs include the control by nucleotidyl transferase (the enzyme responsible for the addition of the 3'-CCA tail) of specific uterine proteins in rats (Lutz and Barker, 1986) and ubiquitin-ATP-dependent protein degradation (Ferber and Ciechanover, 1986).

A rather unusual characteristic of tRNA is the finding that certain patients with systemic autoimmune disease can, in some cases, direct the production of antibodies against the initiator methionine tRNA (Wilusz and Keene, 1986) as well as the anticodon region of tRNA^{Ala} in the autoimmune disease myositis (Bunn and Mathews, 1987). These disease processes indicate the importance of a detailed understanding of tRNAs and their respective genes, since they are involved in more than protein synthesis and their analysis may provide useful insights into the nature of certain diseases in humans.

(c) Organization of tRNA genes in *Escherichia coli*

Transfer RNA genes in *E. coli* are located at various points in the genome, sometimes in multiple copies. They occur in three types of transcriptional formats: i) tRNA genes only, singly or in clusters, ii) tRNA genes and the three ribosomal RNA genes and iii) tRNA genes and protein encoding genes (Fournier and Ozeki, 1985).

Several tRNA gene clusters have been identified in the *E. coli* genome. For example, Egan and Landy (1978) described a tRNA^{Tyr} gene cluster which encoded two copies of the same gene. The *leuS* locus contains seven tRNA genes specifying methionine, leucine and glutamine acceptors (Nakajima *et al.*, 1981). There are also operons which encode lysine-valine-lysine, arginine-histidine, histidine-leucine-proline and leucine-leucine-leucine tRNAs (Yoshimura *et al.*, 1984; Hsu *et al.*, 1984; Duester *et al.*, 1981).

Each of the seven rRNA operons in *E. coli* contains at least one tRNA gene between the 16S and 23S rRNA genes. The tRNA genes in *rrnA, D* and *H* specify acceptors of isoleucine and alanine. Glutamate tRNA genes can be found within *rrnB, C, E* and *G*. The operon *rrnD* also contains asparagine and tryptophan tRNA genes at its distal end. Threonine and asparagine tRNA genes can be found at the distal ends of *rrnD* and *rrnH,* respectively (Ellwood and Nomura, 1982; Morgan *et al.,* 1980).

There are also examples of tRNA genes adjacent to protein genes. Two of these operons have been shown to encode known proteins and tRNAs. The genes for three isoaccepting species of $tRNA^{Thr}$ and one $tRNA^{Gly}$ can be found in close association with the elongation factor Tu (Lee *et al.,* 1981; Miyajima *et al.,* 1981). The *metY* locus, located at approximately 68 map units, encodes a $tRNA^{Met}$ gene as well as the complete coding region of the *nusA* 54.4 Kd protein (Ishii *et al.,* 1984).

(d) Organization of tRNA genes in eukaryotic organisms

In eukaryotic organisms tRNA genes can be found as members of complex multigene clusters which are believed to be located throughout the genome. Alternatively single tRNA genes of the same acceptor specificity can usually be found at other locations in the genome. The purpose, if any, of these organizational patterns is not known, although the theory has been put forth that this redundancy of specific tRNA genes may be important in the synthesis of tRNA species which may be needed during set times in the cell cycle (for a recent review of eukaryotic tRNA genes see Sharp *et al.,* 1985). Clustering may serve as a means to synthesize related gene products from otherwise independent transcription units.

Approximately 350 tRNA genes have been estimated to exist in the genome of *Saccharomyces cerevisiae.* These genes do not appear to be organized into multigene families (Guthrie and Abelson, 1982). For example, there are eight tRNA^{Tyr} genes which are not linked but are dispersed throughout the genome (Olson *et al.,* 1977). Yeast would appear to be an exception among eukaryotic organisms, in which clusters can generally be found. Schmidt *et al.* (1980) however, have reported a single example of a tRNA gene cluster in yeast, in which arginine and asparagine tRNA genes are located within 5-bp of each other. Similar pairing of tRNA genes has been observed in *Schizosaccharomyces pombe* (Gamulin *et al.,* 1983; Mao *et al.,* 1980). These tandem genes, in contrast to tRNA gene clusters of higher eukaryotic organisms, are transcribed together rather than individually. Reyes *et al.* (1986) have shown that the two tRNA genes in one yeast tRNA^{Arg}-tRNA^{Asp} gene tandem can each be independently transcribed if an inhibitory sequence is removed from between the two genes (see also Straby, 1988).

Although there is evidence that tRNA genes may occur throughout a typical eukaryotic genome, the organization apparently is not random. *In situ* hybridization studies with *Drosophila melanogaster* polytene chromosomes showed that this organism's 600 to 800 tRNA genes (representing 60-90 different tRNA species) are clustered at less than 60 sites. These clusters may contain multiple copies of the same gene or genes of different specificity (Weber and Berger, 1976; Steffensen and Wimber, 1971). One particular tRNA cluster at chromosomal region 42A encodes tRNA^{Asn}, tRNA^{Arg} and tRNA^{Lys} which are contained within 46-kb of

DNA. Seventeen tRNA genes are thought to occur in this chromosomal region such that both strands encode different tRNAs (Yen and Davidson, 1980). Other chromosomal sites can be found which contain more than one tRNA gene. Clusters which encode $tRNA^{Leu}$ and $tRNA^{Ile}$, as well as a cluster containing genes for $tRNA^{Val}$, $tRNA^{Ser}$ and $tRNA^{Phe}$ have been found in the *Drosophila* genome (Robinson and Davidson, 1980; Addison *et al.*, 1982). Serine tRNA genes have also been described in clusters in the *Drosophila* genome (Cribbs *et al.*, 1987a; Cribbs *et al.*, 1987b).

A rather unusual situation exists in *Xenopus laevis* where a well characterized population of tRNA genes is contained within a 3.18-kb length of DNA which is repeated at least one hundred times on a single chromosome (Müller and Clarkson, 1980; Fostel *et al.*, 1984). Each repeating unit codes for single species of $tRNA^{Met}$, $tRNA^{Phe}$, $tRNA^{Tyr}$, $tRNA^{Asp}$, $tRNA^{Ala}$, $tRNA^{Leu}$ and $tRNA^{Met}$ and $tRNA^{Lys}$ pseudogenes (Müller *et al.*, 1987). Since this DNA sequence is repeated to such a high extent, it would suggest a special role for these genes, perhaps in oogenesis.

In humans, 1300 tRNA genes have been estimated to occur representing 10 to 20 copies each of 60-90 different species (Hatlen and Attardi, 1971). It is widely assumed that these genes are distributed throughout the genome. Santos and Zasloff (1981), using a *X. laevis* $tRNA_i^{Met}$ gene probe, have shown that the human genome has at least 12 $tRNA_i^{Met}$ genes at separate locations in the genome. Similarly, members of one $tRNA^{Val}$ gene family have been shown to occur at 13 separate loci (Arnold *et al.*, 1986). A human $tRNA^{Gly}$ gene exists which is apparently not associated with any other functional tRNA genes, although a pseudogene can be found within 0.8-kb of the functional tRNA^{Gly} gene (Pirtle *et al.*, 1986). A second isoaccepting tRNA^{Gly} gene has also been described which is not associated with other tRNA genes (Shortridge *et al.*, 1985). Other single copy tRNA genes include examples for tRNA^{Tyr} and two identical tRNA^{Asn} genes (van Tol *et al.*, 1987; Ma *et al.*, 1984).

The first completely characterized multigene cluster in humans was described by Roy *et al.* (1982), in which single species of tRNA^{Lys}, tRNA^{Gln} and tRNA^{Leu} genes were found on a 1.65-kb length of DNA (see also Buckland *et al.*, 1983). Goddard and coworkers isolated a cluster of tRNA genes, two of which have been shown to encode tRNA^{Glu} and a previously reported tRNA^{Leu} (Roy *et al.*, 1982; Goddard *et al.*, 1983; McLaren and Goddard,1986). Four tRNA genes, two tRNA^{Pro}, tRNA^{Leu} and tRNA^{Thr} were located within an 8.2-kb length of DNA (Chang *et al.*, 1986). Doran *et al.* (1987) found four tRNA genes on a 13.8-kb length of DNA, two of which were shown to be identical to a previously reported tRNA^{Lys} gene (Roy *et al.*, 1982). The other two genes were the first reported examples of human (and mammalian) tRNA^{Phe} genes. At least four tRNA genes encoding tyrosine-accepting tRNA species occur on a 9.2-kb DNA fragment (see RESULTS). There is no evidence, as yet, to suggest that the human genome contains tandemly repeated clusters of tRNA genes, as has been shown with *X. laevis*, or that the genes are confined to relatively few cytological loci, as is the case with *Drosophila* tRNA genes. The functional significance, if any, of these varied organizational patterns is not known.

(e) Transcription of eukaryotic tRNA genes

The eukaryotic tRNA gene transcriptional unit is predominantly monomeric such that each gene is transcribed independently no matter how close adjacent genes may be. There does not appear to be any significant conserved 5'-flanking region which can act as a promoter. All eukaryotic tRNA gene promoter sequences are located within the gene (DeFranco *et al.*, 1980; Hofstetter *et al.*, 1981). The DNA sequence responsible, at least in part, for transcription initiation by RNA polymerase III is contained within two separate regions of the gene termed the A and B blocks (Galli *et al.*, 1981). The A block is positioned in the area which codes for the D-loop (also called the D-control region or the 5'-internal control region) and the B block is found within the T-loop (the T-control region or the 3'-ICR). These two regions are highly conserved in all tRNAs, both bacterial and eukaryotic. It has been suggested that the A block may function in the correct placement of RNA polymerase III (Ciliberto *et al.*, 1983; Sharp *et al.*, 1983) and the B block has a role in the binding of transcription factors. There appears to be a limit to the separation between these two promoter regions (Sharp *et al.*, 1983; Dingermann *et al.*, 1983). This view may be rather simplistic since there are at least two factors (proteins) involved in the transcription of these genes, as well as putative sequences outside the genes which have a major role in transcription regulation.

In those cases extensively studied, there is increasing evidence that the 5'-flanking sequences of some tRNA genes have an effect on the level of transcription (DeFranco *et al.*, 1980; Sprague *et al.*, 1980; Cooley *et al.*, 1984). In one case, a silkworm tRNA^{Ala}

gene absolutely requires its 5'-flanking region for expression in a homologous *in vitro* transcription system (Sprague *et al.*, 1980). The *X. laevis* variant $tRNA^{Met}$ gene, however, appears to contain both strong and weak inhibitory sequences located within the adjacent 124 nt of it's 5'-flanking region (Hipskind and Clarkson, 1983). Dingermann *et al.* (1982) have also shown that one *Drosophila* $tRNA^{Arg}$ gene contains five consequative 'T's in it's 5'-flanking region which completely inhibits the expression of this gene in a homologous *in vitro* transcription system. A common 11-bp sequence can be found within the 5'-flanking region of the known $tRNA^{Lys}$ genes of *Drosophila*, and yet the genes are not transcribed with the same efficiency. DeFranco and coworkers (1981) have shown that the position of this specific sequence has a direct effect on the transcription activity of these genes. A similar situation exists with the yeast $tRNA_3{}^{Leu}$ gene (Raymond and Johnson, 1983; Johnson and Raymond, 1984). A $tRNA^{Tyr}$ gene from yeast was examined by deletion analysis, and it was shown that the first 60-bp immediately preceding this gene were required for activation of the gene, although some products could be observed with deletion mutants which had less of the normal 5'-flank (Shaw and Olson, 1984). A similar situation was found with a *Drosophila* $tRNA_4{}^{Val}$ gene, in which a positive element can be removed in the 5'-flanking region. This resulted in a 100-fold decrease in the expression of this particular gene (Sajjadi *et al.,* 1987). Three subcloned *Drosophila* $tRNA_5{}^{Asn}$ genes have been used to examine directly the importance of the 5'-flanking regions of these genes. Exchanging 5'-flanking regions between these three tRNA genes showed that the differences in the noted levels of transcription were due to the sequence present in front of each gene (Lofquist and Sharp, 1986). Thus, the 5'-flanking region appears to have a direct influence on the level of transcription of some but not all tRNA genes.

In addition to the ICR's and 5'-flanking regions, there may also be a dependence upon the 3'-flanking region for correct expression of some tRNA genes. It is known that the transcription termination signal is located in the 3'-flanking region of the gene and consists of four or more consequative 'T' residues (Bogenhagen *et al.*, 1980). A recent study with a human $tRNA_i^{Met}$ gene indicated that this gene does not have a sequence of four or more 'T' residues, and as a result, three 'run-on' transcripts were observed from this gene (Vnencak-Jones *et al.*, 1987). It now appears that this region does more than simply terminate gene transcription. Using a competition assay dependent upon the ability of tRNA genes to form stable transcription complexes, Schaack and colleagues (1983; 1984) have shown that at least 35 nt of the 3'-flank are required for stable transcription complex formation on a *Drosophila* $tRNA^{Arg}$ gene.

Wilson *et al.* (1985), using a silkworm $tRNA_2^{Ala}$ gene, have also demonstrated that its flanking regions are important for transcription of this particular gene. It became apparent that there may have been an inhibitory factor in their cell extracts which could account for the great variability in transcription observed. This could suggest that all tRNA genes have a complex transcription control mechanism that involves several regions: the clearly defined ICRs, the 5'-flanking regions and the 3'-flanking regions. Consistent with this theory is the observation that the entire coding region of a yeast $tRNA^{Leu}$ gene, as well as some 5'- and 3'-flanking sequences, are protected from nuclease digestion by yeast transcription factors (Klemenz *et al.*, 1982; Stillman and Geiduschek, 1984; Stillman *et al.*, 1984). Therefore it is necessary to obtain a better understanding of the transcription factors involved in tRNA gene expression.

(f) Protein factors involved in eukaryotic tRNA gene transcription

The transcription of eukaryotic tRNA genes is dependent upon RNA polymerase III (Roeder and Rutter, 1969). At least two additional protein components are also required, TFIIIB and TFIIIC. These transcription factors have been isolated from human cells (Segall *et al.*, 1980). Similar factors have been discovered in all eukaryotic systems examined to date, including those for *Xenopus, Drosophila* and yeast (Shastry *et al.*, 1982; Burke and Söll, 1985; Taylor and Segall, 1985). DNA protein binding experiments have shown that TFIIIC interacts with both the 3'- and 5'-ICRs (Stillman *et al.*, 1985; Camier *et al.*, 1985; Carey *et al.*, 1986). Yoshinaga *et al.* (1987) have demonstrated that TFIIIC from human cells can be separated into two active components, TFIIIC1 and TFIIIC2. TFIIIC2 binds strongly to the B block, while TFIIIC1 has a weak affinity for the A block. Similar results have been reported (Ottonello *et al.* 1987) for TFIIID isolated from *Bombyx mori* cells, and it may be analogous to TFIIIC2 from human cells. In both cases, binding of the weaker affinity molecule, TFIIIC1 or TFIIIC, is dependent upon the presence of TFIIIC2 or TFIIID, respectively.

The exact function of TFIIIB is not clear although it is necessary for stable complex formation between RNA polymerase III and TFIIIC (Lassar *et al.*, 1983). Burke and Söll (1985) have shown that a *Drosophila* $tRNA^{Arg}$ gene binds TFIIIC rapidly but this association is only stable in the presence of TFIIIB. It is interesting to note that HeLa cell factor TFIIIB is able to interact with *Drosophila* KC cell factor TFIIIC, but the KC cell factor TFIIIB does not produce transcription products when HeLa cell factor TFIIIC is used in place of the homologous component. This would suggest that, although eukaryotic tRNA genes are transcribed by similar processes, the structural components may have definite differences and this could explain why heterologous cell extracts show differential gene expression.

The current model of tRNA gene transcription is complex, involving the transcription factors and RNA polymerase III. TFIIIC interacts with the 3'-ICR (Schaack *et al.*, 1983), and this complex is stabilized by TFIIIB (Lassar *et al.*, 1983). DNAse I protection studies with yeast tRNA genes suggest that there may be at least one more factor involved, as a protein bound to the 5'-ICR had an effect on the overall stability of the transcription complex (Stillman *et al.*, 1984). RNA polymerase III can then recognize the stable complex and begin transcription. These additional transcription factors have not been highly purified. An alternative model has been presented in which it has been shown that, in the absence of DNA template, RNA polymerase III forms an active multisubunit complex which is capable of initiating transcription (Burke and Söll, 1985; Dingermann *et al.*, 1983; Wingender *et al.*, 1986). There is increasing concern that the salt concentrations used in various experiments may have a profound effect on the levels of tRNA gene transcription. Salt dependence has been observed for two silkworm $tRNA^{Ala}$ genes, a yeast $tRNA^{Leu}$ gene, and three *Drosophila* $tRNA^{Asn}$ genes (Young *et al.*, 1986; Raymond and Johnson, 1983; Lofquist and Sharp, 1986). Thus far no complete model of tRNA gene transcription has been developed which explains the varied observations of transcription factor interactions with the ICRs of eukaryotic tRNA genes.

(g) Maturation of eukaryotic tRNA

Eukaryotic tRNA processing involves a series of complex orderly events in which the primary transcript is acted upon by a number of processing enzymes before the RNA becomes a mature tRNA molecule. Detailed studies with yeast $tRNA^{Tyr}$ genes have led to an increased understanding of the processing of eukaryotic tRNAs (DeRobertis and Olson, 1979). The primary transcript consists of an RNA molecule which has a 5'-leader, the sequence complementary to the tRNA gene, and some 3'-flanking sequence down to and including the tract of U residues corresponding to the termination sequence of the gene. First the 5'-leader is removed in at least two stages, resulting in the mature 5'-end of the tRNA. This processing appears to be accomplished by an enzyme similar to RNase P in *E. coli* (Koski *et al.*, 1976; Kline *et al.*, 1981; Engelke *et al.*, 1985). Then the 3'-tail is removed by a 3'-endonuclease as in yeast (Engelke *et al.*,1985; Pearson *et al.*, 1985) or by a 3'-pre-tRNAase as in *Drosophila* (Frendeway *et al.*, 1985) and *X. laevis* (Castano *et al.*, 1985). Before the addition of the 3'-CCA terminal sequence some base modifications can occur during this processing step. The terminal 3'-CCA tail is added by the enzyme nucleotidyl transferase. After the addition of the 3'-CCA tail, the intron is removed, if the gene has one, and the remaining base modifications are added to obtain the mature tRNA. Although this general scheme applies to transcripts of yeast tRNA genes in an *X. laevis* transcription system, recent evidence suggests that this may not be the case with all eukaryotic tRNA genes. Rooney and Harding (1986), using a mouse $tRNA^{His}$ gene in a HeLa cell extract, showed that this tRNA is processed such that the 3'-tail was removed before the 5'-leader sequence. The only other reported deviations from the described processing order came from studies with multimeric transcripts or in mutated tRNA genes which cannot assume the traditional secondary structure of mature tRNAs (Engelke *et al.,* 1985; Castagnoli *et al.,* 1982). Tocchini-Valentini *et al.* (1982) reported that a yeast $tRNA_3^{Leu}$ which cannot form the D-stem failed to have it's 5'-leader removed during processing. In *Xenopus* oocytes, an endonuclease specific for tRNA transcripts which have had their 5'-leader regions removed processes the 3'-tail (Castano *et al.,* 1985). In humans, however, the removal of the intervening sequence does not occur at the same stage of processing as has been observed with other eukaryotic systems. The intervening sequence is removed before maturation of the 5'- and 3'-ends of the pre-tRNA (van Tol and Beier, 1988).

(h) Intervening sequences in tRNA genes

An added complexity of tRNA processing is the fact that some tRNA genes are interrupted by intervening sequences. Intervening sequences have been found in all eukaryotic tRNA^{Tyr} genes examined (Goodman *et al.,* 1977; Müller and Clarkson, 1980: Kubli *et al.,* 1988; MacPherson and Roy, 1986). Their role in tRNA gene regulation, if any, is not understood. Introns in tRNAs usually occur one base 3' to the anticodon, although variations are known (Del Rey *et al.,* 1982; Ogden *et al.,* 1984). They usually contain a sequence which is complementary to the anticodon (Johnson and Abelson, 1983). An exception to this general observation is a solitary tRNA^{Tyr} gene from *X. laevis* (Gouilloud and Clarkson, 1986). Despite the fact that a definite function for most introns remains obscure, Johnson and Abelson (1983) have shown that a yeast tyrosine tRNA gene intron is required for a specific modification of the tRNA. When the intron from this gene was removed, by site-directed mutagenesis, a uridine to pseudouridine modification in the anticodon of the tRNA failed to occur, and a concomitant decrease in the expression of the gene was observed.

The mechanism(s) by which these introns are removed is not well defined. As discussed above, precursor tRNAs may be processed along different pathways (Greer *et al.,* 1983; Filipowicz and Shatkin, 1983). The splicing of introns has been studied most extensively in yeast, in which the enzymes involved have been partially purified (Greer *et al.,* 1983; Peebles *et al.,* 1983). The splicing endonuclease, *Xla*I RNase, has also been purified from *X. laevis* (Attardi *et al.*, 1985). Since a single enzyme appears to be responsible for the cleavage of all known intron-containing tRNAs in yeast, this could suggest that the secondary structure of these molecules might be important for the recognition of the correct splice site. Lee and

Knapp (1985) have examined the structure of four yeast tRNAs by partial nuclease digestion experiments. It was found that the cloverleaf structure was maintained in the precursors and that the anticodon loop was interrupted by the intron. This intron contained a region which base paired with the anticodon to produce an extended anticodon stem-loop structure. Swerdlow and Guthrie (1984) have shown that the splice sites appear to be maintained as single-stranded loops. Winey *et al.* (1986) determined that a yeast suppressor pre-tRNA^{Pro} which has an altered anticodon stem that cannot base pair properly with the intron, is not spliced as efficiently as the wild type pre-tRNA. Dingermann *et al.* (1988) obtained similar results with a *Dictyostelium* tRNA^{Trp} gene whose transcription products increase if the intron, which cannot base pair with the anticodon, is removed. Thus, introns appear to affect the maturation of precursor tRNAs which contain them.

(i) Eukaryotic tRNA^{Tyr} genes

The sequences of tRNATyr genes from some multicellular organisms are highly conserved (Müller and Clarkson, 1980; van Tol *et al.,* 1987; Kubli *et al.,* 1988; MacPherson and Roy, 1986). A detailed study of *X. laevis*, tRNA^{Tyr} genes and three human tRNA^{Tyr} genes revealed little sequence homology between their flanking regions (Müller and Clarkson, 1980; van Tol *et al.,* 1987; MacPherson and Roy, 1986). Similar observations have been reported for other tRNA gene families (Arnold *et al.,* 1986). Considering the sequence homology within the structural genes it seems surprising that there is such low conservation of flanking sequence. A *X. laevis* tRNA^{Tyr}

gene which is a single-copy gene has no flanking sequence homology with a tRNA^{Tyr} gene in the 3.18-kb gene cluster, although the coding sequences are almost identical (Gouilloud and Clarkson, 1986). There are, however, examples of substantial sequence homology between the 5'-flanking regions of two human tRNA^{Tyr} genes (MacPherson and Roy, 1986). Santos and Zasloff (1981) reported limited sequence homology between the 5'-flanking regions of two human tRNA$_i^{Met}$ genes. One example has been shown of sequence homology between organisms, in which the 5'-flanking region of a mouse tRNA$_i^{Met}$ gene shares patchwork homology with that of a human tRNA$_i^{Met}$ gene (Han *et al.,* 1984). van Tol *et al.* (1987) reported a tobacco plant tRNA^{Tyr} gene that has some sequence homology with a human tRNA^{Tyr} gene. It is interesting to note that the gene reported for the human tRNA^{Tyr} by these workers shares no 5'- or 3'-flanking region similarity to the genes reported by MacPherson and Roy (1986). Thus, there is as yet no evidence to suggest that human tRNA genes are contained in tandemly repeated clusters as is the case with certain *X. laevis* tRNA genes (Müller and Clarkson, 1980).

The tRNA^{Tyr} gene reported for *X. laevis* has an intron of 13-bp which begins immediately after the anticodon (Müller and Clarkson, 1980). In the human tRNA^{Tyr} genes, the introns are 21-bp and 20-bp in length, begin one base 3' to the anticodon and have 30 to 100% sequence homology (MacPherson and Roy, 1986; van Tol *et al.,* 1987). Introns have also been reported to occur in *Drosophila* tRNA^{Tyr} genes and have three different size classes (Kubli *et al.,* 1988). Six tRNA^{Tyr} genes have introns of 20 or 21-bp, one gene has a 48-bp intron and another gene contains a 113-bp intervening sequence. Thus there appear to have been evolutionary constraints to maintain a unique sequence in those genes which have introns of 20 or 21-bp since these introns have considerable sequence homology. Since *Drosophila* has at least three size classes of introns within its $tRNA^{Tyr}$ genes it is interesting to speculate that humans will also have varying lengths of intervening sequences.

Since only a limited number of the estimated 1300 human tRNA genes have been described, a search was done to isolate human $tRNA^{Tyr}$ genes by screening a human DNA library. Transfer RNA^{Tyr} genes were selected because it was suspected that they would contain intervening sequences. This dissertation describes the isolation and characterization of six human $tRNA^{Tyr}$ genes, and the nucleotide sequence of five of them. The expression of these genes in a homologous *in vitro* transcription system was also investigated, and remarkable differences in the rates of transcription were observed.

MATERIALS AND METHODS

Materials

(a) Strains and media

A human-λ Charon-4A phage recombinant library was a gift of Dr. T. Maniatis (Lawn *et al.*, 1978). *Escherichia coli* DP50*supF* was used for propagation of these recombinant phage (Leder *et al.*, 1977). *E. coli* HB101 (Boyer and Roulland-Dussoix, 1969) was used for the propagation of the plasmid pAT153 recombinant subclones (Twigg and Sherratt, 1980). *E. coli* MV1193, JM103, or JM105 were used for the propagation of recombinant M13 phage DNA (Yanisch-Perron *et al.*, 1985). Agar, yeast extract, and Bacto tryptone were from Difco Laboratories. TYDTM medium was used as the growth medium for *E. coli* DP50*supF* (Leder *et al.*, 1977). *E. coli* HB101 was grown in LB medium and *E. coli* MV1193, JM103, or JM105 were propagated in YT medium (Maniatis *et al.*, 1982). The bacterial strains were stored by mixing an equal volume of glycerol to mid-log phase cells and freezing at -80°C. Recombinant phage were stored in SM buffer over chloroform at 4°C. Plasmid stocks were stored in TE buffer at -20°C.

(b) Laboratory materials and enzymes

Nitrocellulose filters were from Schleicher and Schuell or Millipore; Gene Screen Plus membranes were from DuPont. X-ray film was Kodak XAR-5 or Konica medical X-ray film. Chemicals were reagent grade. SeaKem agarose was procured from Mandel Scientific Company. Acrylamide was from Bethesda Research Laboratories or

Eastman Kodak Company, while N,N'-methylenebisacrylamide was from BDH Chemicals. Restriction endonucleases, T4 DNA ligase, and *E. coli* DNA polymerase I from New England Biolabs, Pharmacia, Boehringer-Mannheim or Bethesda Research Laboratories were used as specified by the manufacturers. New England Nuclear or Amersham supplied [α-^{32}P]dATP, [γ-^{32}P]ATP and [α-^{32}P]GTP (3000 Ci/mmole). Deoxyribonucleoside triphosphates and ribonucleoside triphosphates were from Sigma or Pharmacia. The 17-bp universal sequencing primer, number 0985-04 was purchased from the Regional DNA Synthesis Laboratory, University of Calgary, or Pharmacia.

(c) *In vitro* transcription extracts

In vitro transcription reactions were performed using Bethesda Research Laboratories eukaryotic *in vitro* transcription system lot number 72101 as instructed by the supplier, or as described by Manley *et al.,* (1980).

Methods

Growth and isolation of human-λ Charon-4A recombinant phage containing tRNA genes

(a) Determination of plaque forming units in the human-λ recombinant phage library

The human DNA library was constructed from partial *Hha*I-*Alu*I digestion products of human placental DNA which were inserted into the bacteriophage vector λ-Charon-4A (Lawn *et al.,* 1978). Since the recombinant library was in limiting amounts, a secondary amplification, previously prepared by Dr. T. Maniatis, was used as starting material. Ten µl of the library were diluted with 10 ml of SM buffer (50 mM Tris-HCl, pH 7.8, 8 mM $MgCl_2$, 0.1% gelatin). From this dilution, 10 µl were used to infect 100 µl of exponentially growing *E. coli* DP50*supF* cells. After a 10 min incubation at 37°C, 50 µl of 2% X-GAL and 10 µl of 100 mM IPTG were added. Three ml of 0.7% agarose-TYDTM (1% tryptone, 0.5% yeast extract, 86 mM NaCl, 0.1% casamino acids, 0.01% diaminopimelic acid, 0.004% thymidine and 50 µg/ml nalidixic acid, which maintained the purity of the culture because the bacterial strain is Nal^r) medium at 46° C were mixed into the virus-cell suspension, and this was poured over prewarmed (37°C) TYDTM agar plates and allowed to cool for 30 min. Plaques formed overnight during incubation at 37°C. The following day the plates were scored for white plaques, indicating recombinant virus, and for total viable virus per µl of the human λ-recombinant library.

(b) Large scale screening of the recombinant phage library

Screening of the human-λ Charon-4A recombinant phage library was as described by Benton and Davis (1977). Ten µl of the recombinant phage library (2.2 X 10^9 pfu/ml) were diluted to 100 µl with SM buffer. From this dilution 15 µl aliquots were added to 0.5

ml portions of exponentially growing *E. coli* DP50 *supF* cells and incubated at 37°C. After 20 min, 25 ml of 0.7% agarose-TYDTM medium at 46°C were added to these and the suspended cells were spread over 23 cm by 23 cm Petri plates containing prewarmed (37°C) TYDTM agar. These plates were cooled for at least 30 min and then incubated for 12 to 16 hr to allow plaque formation. After cooling for at least one hr at 4°C, a 20 cm by 20 cm nitrocellulose filter was placed directly onto the surface of each plate and left for 1 min to allow the adsorption of phage from each plaque to the filter. The filter was peeled off the plate and floated on a solution containing 0.1 M NaOH, 1.5 M NaCl for 1 min, then floated on 0.2 M Tris-HCl pH 7.8, 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.8) for 5 min to neutralize the filter. The membrane was dried and baked under vacuum for 2 hr at 80°C.

(c) Preparation of ^{32}P-labeled pXt267 DNA

The probe used to detect human tyrosine tRNA genes was pXt267, a plasmid containing a 267-bp *Hha*I DNA fragment, encoding a tRNA^{Tyr} gene from the 3.18-kb *X. laevis* tRNA gene cluster (Müller and Clarkson, 1980), which had been subcloned, using *Hin*dIII linkers, into pAT153 (Lam, W. and Roy, K. L., unpublished). This plasmid was nick-translated as described by Rigby *et al.* (1977). Approximately 350 ng of pXt267 DNA were preincubated at 15°C for 10 min in a total volume of 27.5 µl of NT buffer (50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 2.5 mM dithiothreitol) in the presence of 3.3 µM each of dCTP, dGTP, dTTP and 10 µCi [α-^{32}P]dATP (3000 Ci/mmol). After the preincubation, DNAse I (1.0 ng) and *E. coli* DNA polymerase I (0.3 u) were added and the mixture was incubated for 1 hr at 15°C, to yield a probe with approximately 4 x10^7 dpm. The labeling reaction was stopped by the addition of EDTA (pH 8.0) to a final concentration of 5 mM. The probe DNA was stored at -20°C for less than one week if not used immediately.

(d) Hybridization of ^{32}P-labeled pXt267 DNA to DNA from the recombinant phage library

The hybridization of ^{32}P-labeled pXt267 DNA was as described by Maniatis *et al.* (1982). The nitrocellulose filters with bound recombinant phage DNA were placed inside Sears Seal-a-Meal bags and were incubated overnight in 8 ml of hybridization buffer (6 x SSC, 0.5 mg/ml Ficoll 400, 0.5 mg/ml bovine serum albumin, 0.5 mg/ml polyvinylpyrrolidone; referred to as Denhardt's solution) and 0.1 mg/ml of heat denatured (90°C for 3 min) *E. coli* B DNA at 65°C with agitation. After prehybridization, the ^{32}P-labeled pXt267 DNA was heat denatured and added to 9 ml of hybridization buffer. The probe solution was added to each filter being tested, and hybridization was allowed for 16 hr at 65°C. Following the hybridization, the filters were washed in two changes (2 l each) of 2 x SSC, 0.1% SDS for 30 min at 65°C and 2 changes (2 l each) of 0.2 x SSC, 0.1% SDS for 30 min at 65°C. The filters were air dried and autoradiographed at -70°C with Dupont Lightning Plus intensifying screens.

(e) Plaque purification of recombinant phage containing tRNA^{Tyr} genes

Plaques showing hybridization to the *X. laevis* tRNA^{Tyr} gene probe were purified for further characterization. Each desired plaque was extracted from the agarose overlay with a sterile Pasteur pipette. The narrow end of a Pasteur pipette was stabbed into the plate and an agarose plug containing the desired plaque and underlying medium was removed and placed in 1 ml of sterile SM buffer containing 100 µl of chloroform. The phage particles within the plug were allowed to diffuse into the SM buffer overnight at 4°C. This procedure routinely produced a phage suspension of approximately 5 x10^6 pfu/ml.

Isolated recombinant bacteriophage were plaque-purified essentially as described above, with the exception that this was done using standard Petri plates. Stocks of each recombinant bacteriophage were made using the plate lysate procedure (Maniatis *et al.*, 1982). Conditions were such that confluent lysis occurred on a lawn of *E. coli* DP50*supF* cells after infection and overnight growth at 37°C. A 100-fold dilution of the isolated and purified recombinant phage was used to infect exponentially growing *E. coli* DP50*supF* cells as described above. After growth overnight, 5 ml of sterile SM buffer was poured onto the plate of lysed bacterial cells and this was left at 4°C for 24 hr to allow phage particles to diffuse into the SM buffer. The phage suspension was collected with a sterile Pasteur pipette. This procedure gave phage suspensions of approximately 2-4 x10^{12} pfu/ml.

(f) Large scale purification of recombinant phage

Isolated bacteriophage clones encoding $tRNA^{Tyr}$ genes were amplified as described by Maniatis *et al.* (1982). The OD_{600} of a 3 hr culture of *E. coli* DP50*supF* cells was determined, and four aliquots containing 10^{10} cells were centrifuged at 2000 x g for 10 min at 4°C. Each cell pellet was resuspended in 3 ml of sterile SM buffer and 5 $\times 10^{9}$ bacteriophage particles were added to each cell suspension and incubated at 37°C for 30 min to infect the cells. Each of the four infected *E. coli* DP50*supF* samples was added to 500 ml of prewarmed (37°C) TYDTM broth and incubated for 8-10 hr with rapid shaking at 37°C. The OD_{600} was determined every hour during growth to detect when cell lysis had occurred. After lysis, 10 ml of chloroform were added to each 500 ml culture and incubation at 37°C with rapid shaking was continued for a further 10 min to complete cell lysis. The lysates were cooled to room temperature and 2.5 ml of RNase A (0.2 mg/ml) were added to each 500 ml culture. This was incubated for 30 min at room temperature after which time NaCl was added to a final concentration of 1 M and this was incubated on ice for 1 hr.

The lysates were centrifuged at 11000 x g for 10 min and the phage-containing supernatant was collected. Polyethylene glycol (MW 8000) was added to each preparation to a final concentration of 10% (w/v), and then left overnight at 4°C. The samples were centrifuged at 11,000 x g for 1 hr at 4°C. The pelleted phage were resuspended in 8 ml of sterile SM buffer on ice. Ten µl of DNase I (1 mg/ml) were added and incubated for 10 min at room temperature. The phage suspension was extracted once with an equal volume of chloroform, and the aqueous phases from all samples were pooled and stored at 4°C. A one-tenth volume was saved as a stock solution. To the remaining bacteriophage suspension, 0.5 g of CsCl was added per ml and this was centrifuged as described by Maniatis *et al.* (1982). After centrifugation, CsCl was removed by dialysis against two changes of dialysis buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA and 10 mM $MgCl_2$). DNA was purified from the bacteriophage clones by extraction with an equal volume of phenol with gentle agitation on a culture tube roller for 1 hr followed by centrifugation at 2000 x g in a SS-34 rotor. The aqueous phase was recovered and extracted with an equal volume of chloroform. To the retained aqueous phase a one-tenth volume of 3 M Na acetate (pH 7.0) was added, and the DNA was precipitated by addition of 3 volumes of ice-cold 95% ethanol and the sample was left at -20°C overnight. The following day, the DNA was pelleted by centrifugation at 7700 x g and the supernatant was discarded. The DNA was redissolved in TE buffer (10 mM Tris-HCl pH 7.8, 1 mM EDTA) to a final concentration of 0.5-1.0 µg/µl and was stored at -20°C.

(g) Restriction enzyme mapping of purified recombinant phage DNAs

A series of single or double restriction enzyme digestions of the recombinant phage DNAs was performed to determine the location of restriction sites within each DNA molecule. Single restriction enzyme digestions were done by digesting 1.5 µg of bacteriophage DNA with 3 u of enzyme in the appropriate buffer (20µl) for 2 hr. After the incubation, usually at 37°C, the resulting DNA fragments were separated by electrophoresis on a 0.75% agarose gel at 5 V/cm in TEA buffer (0.02 M Tris-HCl, pH 7.8, 2 mM EDTA and 0.05 M sodium acetate). After completion of electrophoresis the DNA fragments were stained with ethidium bromide and visualized with UV illumination. If the gel was to be used for hybridization analysis, it was transferred to nitrocellulose or Nylon (Gene Screen Plus) membranes as described by Southern (1975).

Double digestions of the bacteriophage DNAs with two different restriction endonucleases were preformed by first digesting 10 µg of bacteriophage DNA with 20 u of one enzyme in 50 µl of the appropriate buffer for two hr at 37°C. After complete digestion the DNA was precipitated at -70°C for 10 min. The DNA was then centrifuged for 10 min in an Eppendorf centrifuge at 4°C. The supernatant was removed with a drawn out Pasteur pipette, and the sample was dried under vacuum. The sample was redissolved in 10 µl of double distilled water (ddH$_2$O). Two µg of the digested DNA was cut with a second enzyme in 20 µl using 3-4 u of enzyme for 2 hr at 37°C. The resulting DNA fragments were then separated by electrophoresis on a 0.75% agarose gel and processed as described above.

(h) Mapping of restriction enzyme sites of ^{32}P-labeled bacteriophage DNA

To define the order of certain restriction enzyme sites within the recombinant phage DNA, the mapping procedure of Smith and Birnsteil (1976) was used. *XhoI* was used because it had a limited number of sites within the recombinant DNAs. Twenty μg of bacteriophage DNA were digested with 40 u of *XhoI* in 50 μl of the appropriate buffer for 3 hr at 37°C. The DNA was then precipitated as described above. The supernatant was removed and the sample was dried under vacuum for 20 min at room temperature. The DNA was then 3'-labeled with 0.5 u of *E. coli* DNA polymerase I (Klenow fragment) in 30 μl of NT buffer, with 3.3 μM each of dCTP, dGTP, dTTP and 5 μCi [α-^{32}P]dATP (3000 Ci/mmol) for 1 hr at 15°C (Müller and Clarkson, 1980). The [3'-^{32}P]-labeled DNA fragments were electrophoretically separated on a 0.75% agarose gel at 5 V/cm, stained with ethidium bromide, and visualized with UV-illumination. A gel slice containing the desired DNA fragment was excised from the gel. The 3'-labeled DNA was recovered from the gel by the freeze-squeeze method as described by Thuring *et al.*, (1975).

Partial digestions of the labeled DNA fragment were conducted by incubating a 5 μl aliquot of the DNA sample with either 0.1, 0.5, or 1.0 u of the desired enzyme in the appropriate buffer for 30 min at 37°C. The partially digested DNA fragments were then electrophoresed on a 0.75% agarose gel with ^{32}P-labeled DNA size markers at 5 V/cm. After electrophoresis, the gel was soaked in two changes of 7% TCA. The gel was then placed on a sheet of Whatman

3MM paper, and a stack of paper towels was layered on top to dry the gel. After drying overnight the gel was radioautographed at -70°C using Kodak XAR-5 X-ray film.

(i) Hybridization of ^{32}P-labeled rabbit liver tRNA to isolated recombinant phage DNA

Approximately 3 μg of unfractionated rabbit liver tRNA was digested in 50 mM sodium carbonate/bicarbonate (pH 9.0) for 8 min at 90°C. After the incubation the tube was placed in an ice water bath to stop the reaction. Three hundred ng of degraded tRNA were 5'-labeled with [γ-^{32}P]ATP and polynucleotide kinase (Maizels, 1976). The [5'-^{32}P]tRNA was hybridized to a Southern transfer of digested recombinant bacteriophage DNA. The hybridization was conducted in 6 x SSC, 5 x Denhardt's solution, 0.1% SDS and 50% formamide at 46°C for 16 hr with agitation. The filter was washed in 4 x SSC, 0.1% SDS at 40°C for 30 min, then with 2 x SSC at 48°C for a further 30 min. The filter was used to expose Kodak XAR-5 X-ray film at -40°C with Dupont Hi-Speed intensifing screens.

Subcloning tDNA fragments into plasmid vectors

(a) Preparation of a 267-bp *Xenopus laevis* tRNA^{Tyr} gene probe

Since the plasmid used to screen the recombinant phage DNA library was a pAT153 derivative and the DNA fragments to be subcloned were to be ligated into pAT153, the 267-bp probe fragment from pXt267 had to be isolated for use as a unique probe. Digestion with *Hin*dIII released the 267-bp DNA fragment, which contained the coding region for a *X. laevis* $tRNA^{Tyr}$ gene, and this was isolated from a polyacrylamide gel as described by Maxam and Gilbert (1977).

(b) End labeling of the 267-bp DNA fragment

The 3'-recessed ends of the 267-bp *X. laevis* DNA fragment were filled in using the Klenow fragment of DNA polymerase I and a suitable [α-^{32}P] deoxyribonucleoside triphosphate as described by Müller and Clarkson (1980). Approximately 300 ng of the 267-bp DNA fragment were incubated with 0.5 u of DNA polymerase I (Klenow fragment) in NT buffer in the presence of 3.3 µM each of dCTP, dGTP, dTTP and 10 µCi of [α-^{32}P]dATP in 30 µl for 1 hr at 15°C. The reaction was quenched by the addition of 1 µl EDTA (0.5 M, pH 8.0) followed by heating at 70°C for 10 min. The extent of labeling was determined by chromatography on a Sephadex G-50 Superfine column (3 ml packed bed volume) and monitoring with a Geiger counter. The labeled probe was heated to 90°C for 5 min before use.

(c) Subcloning of restriction fragments encoding human tRNAs into plasmid vectors

Recombinant plasmids containing small fragments of human DNA were constructed using standard techniques. Restriction enzyme digestion products of the phage DNAs were subcloned by ligation of total digestion products or of isolated fragments into suitably digested plasmid vector pAT153 (Twigg and Sherratt, 1980). As an illustration, approximately 2 µg of λHtM4 DNA, digested with *Hin*dIII and *Eco*RI, was ligated into pAT153, previously digested with *Hin*dIII and *Eco*RI (1.0 µg). The reaction was conducted in 10 µl of ligation buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 20 mM dithiothreitol and 1 mM ATP) and 0.03 u of T4 DNA ligase overnight at 11°C. Two µl of ligation mixture were used to transform 300 µl of competent *E. coli* HB101 cells as described by Morrison (1979). After a 45 min incubation on ice the cells were heat shocked at 42°C for 2 min, spread over LB plates containing 50 µg/ml ampicillin, and were grown overnight at 37°C.

As the foreign DNA was inserted directly in front of the tetracycline gene of pAT153, colonies were tested in duplicate on LB plates, one containing 50 µg/ml ampicillin and one plate containing ampicillin (50 µg/ml) and tetracycline (12.5 µg/ml). Colonies containing recombinant plasmids could be identified by ampicillin resistance and tetracycline sensitivity. Colony hybridization (Grunstein and Hogness, 1975) was done to identify recombinant plasmids containing human tRNA genes.

(d) Rapid screening of recombinant plasmids

Those plasmids which hybridized to the probe DNA were then examined by the method of Birnboim and Doly (1979) to determine the size of the human insert. Approximately 1 µg of the isolated recombinant DNA was used for restriction enzyme digestion. After a 2 hr digestion at 37°C with the appropriate endonuclease in 20 µl of a suitable buffer, the digested DNA was electrophoresed through a 0.75% agarose gel. The identity of the human DNA insert was determined by direct comparison with the corresponding recombinant phage DNA which had been cut with the same endonuclease.

(e) Large scale preparation of recombinant plasmid DNAs

Large scale isolation of plasmid DNA was essentially as described by Clewell and Helinski (1969). A 10 ml overnight culture of recombinant *E. coli* HB101 cells was added to 500 ml of LB broth at 37°C. The cells were grown with rapid shaking at 37°C until the culture had reached an OD_{600} of 0.5-0.8. Chloramphenicol was added to a final concentration of 170 µg/ml (Clewell, 1972) and the incubation continued overnight. After incubation, the culture was centrifuged at 10400 x g in a GSA rotor for 20 min. The cells were washed once with 50 ml of ice-cold TE buffer and centrifuged as described above.

The cleared lysate was extracted twice with an equal volume of phenol and once with an equal volume of chloroform. The DNA was precipitated with 2.5 volumes of ethanol. The precipitated DNA was redissolved in a small volume of TE buffer and purified by isopycnic banding in CsCl and ethidium bromide as described by Maniatis *et al.* (1982). The isolated plasmid DNA band was extracted repeatedly with 3-4 volumes of butanol to remove the ethidium bromide and the volume of the aqueous phase was maintained with distilled water. Three volumes of ice-cold 70% ethanol were added to precipitate the

DNA and this was left overnight at -20°C. The DNA was pelleted by centrifugation, dried under vacuum, and redissolved in 0.5-1.0 ml of TE buffer. The concentration of the isolated plasmid DNA was determined spectrophotometrically at 260 nm.

(f) Restriction enzyme mapping of plasmid subclones

Recombinant plasmids encoding human tRNA genes were mapped either with four- or six-base specific restriction endonucleases. Routinely, 1-2 µg of recombinant plasmid were digested with 3 u of the desired restriction endonuclease in 20 µl. After 2 hr incubation at the optimum reaction temperature (usually 37°C) the digestion products were separated electrophoretically on 5% or 8% polyacrylamide or 1.0 or 1.25% agarose gels, depending on the sizes of the products. The DNA fragments were then transferred to nitrocellulose or nylon membranes (Southern, 1975) and hybridized to the *X. laevis* $tRNA^{Tyr}$ gene probe to determine which fragments contained human $tRNA^{Tyr}$ genes.

To order the location of the restriction sites within each plasmid, the DNA was end-labeled and mapped (Smith and Birnsteil, 1976) as described above. The partial digestion products of the recombinant plasmids were separated on 30 cm, 1 mm thick, 4% to 8% polyacrylamide gels for varying lengths of time. The radioactive bands were visualized by radioautography at -40°C for 1 to 5 hr with Dupont Hi-Speed intensifing screens.

DNA sequencing and *in vitro* expression of human tRNA genes

(a) Cloning DNA fragments into M13 phage DNA

DNA fragments of less than 500-bp which encoded human tRNA genes were located as described above. These were shotgun-cloned into M13 phage DNA as described above (Messing *et al.*, 1980). Approximately 20 ng of digested plasmid DNA was ligated with 10 ng of M13mp10/11 or M13mp18/19 rf DNA (Yanisch-Perron *et al.*, 1985), which had been digested with an enzyme which produced compatible ends, in 10 µl. After an overnight incubation at 11°C the ligated DNA was either used directly to transfect competent cells or was stored at -20°C for later use.

(b) Transfection of competent *E. coli* cells and detection of recombinant M13 phage

Transfection of competent *E. coli* MV1193, JM103 or JM105 cells was as described by Messing *et al.* (1980). To determine which recombinant M13 DNAs contained human tRNA genes, *in situ* hybridization was done (Benton and Davis, 1977). Immobilized DNA was hybridized to the *X. laevis* tRNA^{Tyr} gene probe as described above. Those plaques which annealed strongly to the probe were transferred to 5 ml of YT broth (Miller, 1972) with a sterile toothpick and incubated on a tube roller overnight at 37°C. One ml of culture was added to a 1.5 ml Eppendorf centrifuge tube and the recombinant phage were isolated as described by Messing *et al.* (1980).

The recovered phage were resuspended in 500 µl of TE buffer. Phage protein was removed by extraction with an equal volume of TE-equilibrated phenol. The resulting aqueous phase was then extracted once with an equal volume of chloroform/phenol (1:1), and the aqueous phase was recovered and the DNA precipitated. After a 1 hr incubation at -20°C the DNA was pelleted by centrifugation and the supernatant was discarded. The DNA pellet was dried under vacuum, and then redissolved in 15 µl of triple distilled H_2O.

(c) Nucleotide sequencing of recombinant M13 DNA

Sequencing was performed using the dideoxynucleoside triphosphate chain termination procedure described by Sanger *et al.* (1977). Approximately 0.5 µg of recombinant M13 DNA was annealed to 5 ng of primer oligonucleotide (Messing *et al.,* 1980) in 10 µl of buffer (66 mM Tris-HCl, pH 7.8, 6.6 mM $MgCl_2$, 6.6 mM dithiothreitol) in a 0.5 ml Eppendorf centrifuge tube by heating to 90°C for 3 min and allowing the tube to slowly cool to room temperature. After the sample had cooled for 30 min, 1 µl of 0.25 M dithiothreitol, 1 µl of [α-^{32}P]dATP (5-10 µCi), 1 µl *E. coli* DNA polymerase I (Klenow fragment, 0.5 u), and 4 µl of double distilled H_2O were added. Four µl aliquots of the reaction mixture were added to 1 µl portions each of four specific nucleoside triphosphate mixtures and incubated at 37°C for 15 min. One µl of chase mix (0.1 mM dNTPs, 0.1 u of *E. coli* DNA polymerase I, Klenow fragment) was added to each tube and the reactions were incubated for a further 15 min at 37°C. The reactions were terminated by the addition of 10 µl of sequencing dye mix (88% formamide, 0.1% bromphenol blue, 0.1% xylene cyanol and 10 mM NaOH). The reactions were stored at -20°C between gel electrophoresis trials.

Gel electrophoresis was carried out as described by Maxam and Gilbert (1977), as modified by Smith and Calvo (1980). Six or 8% polyacrylamide, 8.3 M urea, gels were polymerized in 85 cm, 60 cm or 37 cm length molds. The sequencing gels were pre-run for one hr at the following voltages; the 85 cm gel was set at 3000 V, the 60 cm gel was set at 1700 V and the 37 cm gel was set at 1100 V. The sequencing samples were heated to 90°C for 3 min immediately before application to the gels, and 2 µl aliquots were loaded into each lane of the gel. Electrophoresis was for varying lengths of time to provide overlapping sequences. Radiofluorography at -70°C or -40°C using Kodak XAR-5 or Konica medical X-ray film was performed to visualize the ssDNA products.

(d) *In vitro* transcription of human tRNA genes in a HeLa cell extract

The transcription of human $tRNA^{Tyr}$ genes was examined using homologous extracts prepared as described by Manley *et al.* (1980). One pmol of recombinant plasmid DNA was transcribed in 25 µl of transcription buffer (12 mM Hepes-KOH, pH 7.8, 60 mM KCl, 7.2 mM $MgCl_2$, 0.06 mM EDTA, 1.2 mM dithiothreitol, 5 mM creatine phosphate and 10.2% glycerol) in the presence of 500 µM each of

ATP, CTP, UTP, 50 µM GTP and 10 µCi of [α-^{32}P]GTP at 30°C for 1 hr. The reactions were terminated by the addition of 200 µl of buffer containing tRNA which reduced the extent of exonuclease cleavage of the ^{32}P-labeled human tRNA (7 M urea, 0.3 M sodium acetate, 10 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.1% SDS and 20 µg of *Azotobacter vinelandii* tRNA). One hundred fifty µl of TE-equilibrated phenol was added immediately and vortexed for 15 sec at 4°C. The upper 200 µl of aqueous phase was removed and added directly to 500 µl of ice-cold 95% ethanol, and the solution was mixed by inversion. Precipitation of the RNA products was carried out at -70°C.

The ^{32}P-labeled RNA was pelleted by centrifugation in a microcentrifuge for 20 min at 4°C, and the supernatant was gently removed without disturbing the RNA pellet. The sample was dried under vacuum for 10 min at room temperature and redissolved in 5 µl of loading buffer (88% formamide, 0.1% bromphenol blue, 0.1% xylene cyanol and 1 mM EDTA). The tRNA^{Tyr} gene products were separated by electrophoresis on a 12% polyacrylamide, gel containing 8.3 M urea (Maxam and Gilbert, 1977) at 25 W for 3 hr. The radioactive products were visualized by radioautography at -20°C for 12 hr.

RESULTS

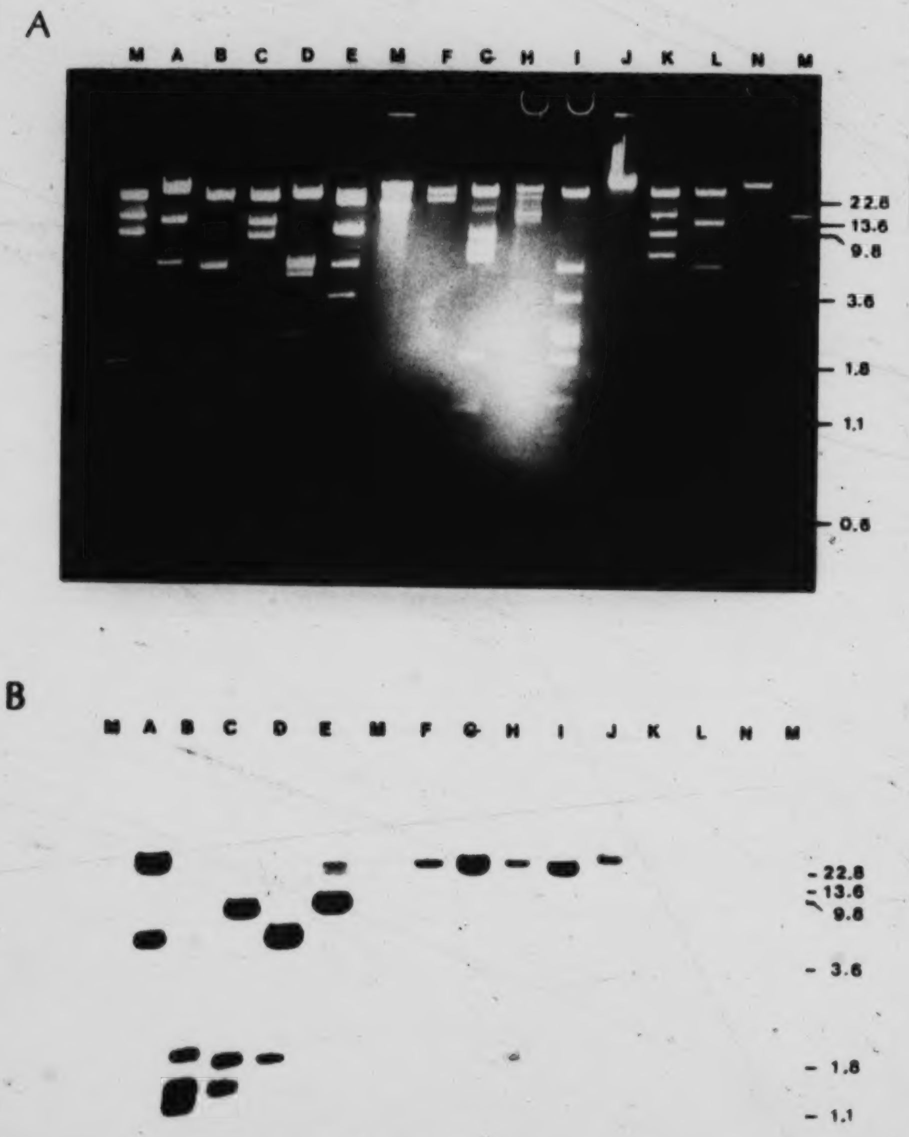
(a) Isolation of tRNA-encoding human-λ Charon-4A recombinant phages

A recombinant human-λ Charon-4A phage library was used in screening for human $tRNA^{Tyr}$ genes. Approximately 200,000 bacteriophage plaques were screened using a *X. laevis* $tRNA^{Tyr}$ gene probe. Seven recombinant bacteriophage clones were isolated which hybridized strongly to the gene probe and these were plaque purified. Plaques were picked for each of the purified, putative, tRNA-encoding bacteriophage clones and were amplified to produce milligram quantities of DNA. Of the seven clones, three failed to amplify and two were found to be identical by restriction mapping (data not shown). As a result, three human-λ Charon-4A bacteriophage recombinant clones were isolated which appeared to encode at least one $tRNA^{Tyr}$ gene. These recombinant bacteriophage were named in sequential order of their isolation as λHtM2, λHtM4 and λHtM6.

(b) Restriction endonuclease mapping of three bacteriophage DNAs

To characterize the isolated bacteriophage clones, the recombinant DNAs were digested with several restriction endonucleases and the resulting fragments were separated by agarose gel electrophoresis and subsequently visualized by UV-illumination. Figure 2A illustrates that these three clones harbored DNA fragments from different regions of the human genome, as the

Figure 2. Restriction endonuclease digestion patterns of three recombinant phage DNAs. (A) Digestions of 2.0 µg of λHtM2, λHtM4 and λHtM6 DNAs were conducted and the products separated on a 0.75% agarose gel. DNA bands were visualized by ethidium bromide-staining and recorded on Polaroid type 665 film. Lanes M are λ-DNA size markers. The left-most marker lane shows λ-DNA digested with *Bgl*II and the right-most marker lane contains λ-DNA digested with *Cla*I. The seventh lane contains λ-DNA which was digested with *Xho*I. Lanes A, B, C, D and E contain DNA (λHtM6) which had been digested with *Xho*I, *Hin*dIII, *Eco*RI, *Bgl*II or *Bam*HI. Lanes F. G, H and I contain λHtM4 DNA which had been digested with *Xho*I, *Hin*dIII, *Eco*RI or *Bgl*II. Lambda HtM2 DNA samples digested with *Xho*I, *Hin*dIII, *Bgl*II or *Bam*HI are shown in lanes J, K, L and N. The DNA samples in lanes G, H, K and N were incompletely digested. (B) Radioautograph of the *X. laevis* [^{32}P]tRNA^{Tyr} gene probe hybridized to a nitrocellulose filter replica of the gel shown in (A). Lane K contains two hybridizing bands of 3-kb and 1.4-kb. The 3-kb band is the result of a partially digested *Hin*dIII fragment of λHtM2. Multiple hybridizing bands were observed from restricted λHtM6 DNA because this clone contained four tRNA^{Tyr} genes.


A
M A B C D E M F G H I J K L N M
22.8
13.6
9.8
3.6
1.8
1.1
0.6
B
M A B C D E M F G H I J K L N M
22.8
13.6
9.8
3.6
1.8
1.1

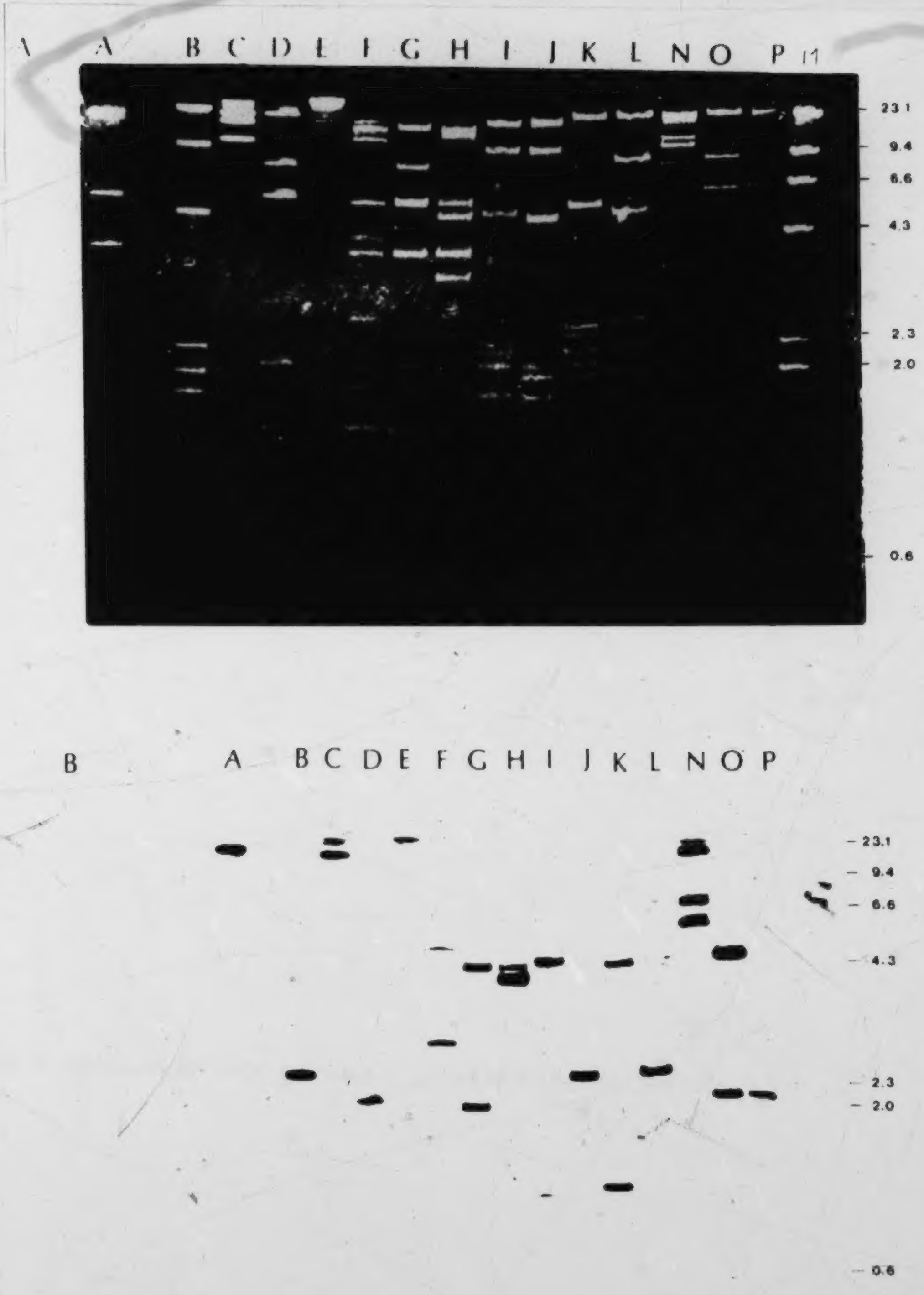
restriction patterns of the digested DNA fragments varied significantly.

Comparison of the mobilities of the recombinant DNA fragments with those of marker fragments showed that λHtM2 has a human DNA insert of 16.0-kb, λHtM4 has a human insert of 16.7-kb and λHtM6 has a human DNA fragment of 15.5-kb. Figure 2B shows hybridization of ^{32}P-labeled pXt267 to fragments of λHtM2, λHtM4 and λHtM6 DNAs which had been immobilized on a nitrocellulose membrane. Both λHtM2 and λHtM4 appear to have at least one putative tRNA^{Tyr} gene since each restriction enzyme digestion produced one hybridizing DNA fragment. Lambda HtM6, however, when digested with *Hin*dIII (lane B of Fig. 2B) shows four DNA fragments of 2.0-kb, 1.5-kb, 1.4-kb and 1.3-kb which hybridized strongly to the pXt267 DNA probe.

To further characterize the clones, the DNAs were singly- and doubly-digested with several restriction endonucleases. Through comparison of the lengths of the resulting fragments the location of restriction enzyme digestion sites could be determined within each bacteriophage DNA.

Lambda HtM2 DNA was mapped using single- and double-digestions with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and *Kpn*I (Fig. 3A). A radioautograph showing the hybridization of a Southern transfer of the same gel to the *X. laevis* tRNA^{Tyr} gene probe is shown in Fig. 3B. The single digestion with *Hin*dIII produced a 2.1-kb DNA fragment which contained a putative tRNA^{Tyr} gene. Digestion with *Bgl*II produced a 2.3-kb DNA fragment which also hybridized to the probe

Figure 3. Restriction endonuclease digestion, Southern transfer and hybridization of λHtM2 DNA. (A) All digestions contained 2 μg aliquots of DNA, which were electrophoresed on a 0.75% agarose gel. Lanes A through E contain *Bam*HI-, *Bgl*II-, *Eco*RI-, *Hin*dIII- or *Kpn*I-digested λHtM2 DNA. Lanes F through I illustrate λHtM2 DNA which had been first digested with *Bam*HI, and then digested with *Kpn*I, *Hin*dIII, *Eco*RI or *Bgl*II. Lanes J, K and L contain λHtM2 DNA which was first digested with *Bgl*II and then digested with *Kpn*I, *Hin*dIII or *Eco*RI. Lanes N and O show λHtM2 DNA doubly-digested with *Eco*RI-*Kpn*I and *Eco*RI-*Hin*dIII, and lane P, *Hin*dIII and *Kpn*I. Lambda DNA digested with *Hin*dIII is in lane M. (B) Radioautograph of a Southern transfer of (A) to which the *X. laevis* $tRNA^{Tyr}$ gene probe DNA had been hybridized. It is assumed that in each case where more than one hybridizing band is observed, the fastest moving band is the complete product and the slower moving bands resulted from incomplete digestion.


A
A B C D E F G H I J K L N O P M
23.1
9.4
6.6
4.3
2.3
2.0
0.6
B
A B C D E F G H I J K L N O P
23.1
9.4
6.6
4.3
2.3
2.0
0.6

DNA. Table 1 shows the sizes of DNA fragments produced from λHtM2 DNA after digestion with several restriction endonucleases.

Figure 4A shows the single- and double-digestion patterns of λHtM4 DNA fragments separated electrophoretically on a 0.75% agarose gel. Probe DNA was hybridized to a Southern transfer of the above digested DNA (Fig. 4B). Table 2 shows the sizes of DNA fragments produced from λHtM4 after having been digested with several restriction endonucleases.

Lambda HtM6 was digested with the restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and *Mlu*I and the resulting DNA fragments were separated on a 0.75% agarose gel (Fig. 5A). A Southern transfer of the fragmented λHtM6 DNA was hybridized to ^{32}P-labeled pXt267 DNA (Fig. 5B). Table 3 indicates the sizes of DNA fragments from λHtM6 after digestion with several restriction endonucleases.

The restriction enzyme *Hin*dIII produced 13 DNA fragments from λHtM6 DNA. Hybridization with the *X. laevis* tRNA^{Tyr} gene probe demonstrated that four of these DNA fragments contained a putative tRNA^{Tyr} gene. Because of the extensive number and small size of the *Hin*dIII generated DNA fragments, the exact location of these fragments within the human DNA insert was difficult to ascertain. To help confirm the physical mapping data, λHtM6 was digested with *Xho* I which generated three fragments of 29-kb, 11.5-kb and 5.9-kb. These DNA fragments were 3'-labeled with [α-^{32}P]dATP and the large fragment of *E. coli* DNA polymerase I. The 29-kb DNA fragment was isolated from a 0.5% agarose gel as described in MATERIALS AND METHODS. This isolated DNA fragment was partially digested with the enzyme *Hin*dIII so that the location of the *Hin*dIII sites

Figure 4. Restriction endonuclease digestion, Southern transfer and hybridization of λHtM4 DNA. The gel was 0.75% agarose and the DNA was visualized by ethidium bromide-staining and UV-illumination. All digestions contained 2.0 μg of DNA. (A) Lanes A through E represent λHtM4 DNA digested with *Kpn*I, *Hin*dIII, *Eco* RI, *Bgl*II or *Bam*HI. Lanes F through I show λHtM4 initially digested with *Bam*HI, and then digested with *Kpn*I, *Hin*dIII, *Eco*RI or *Bgl*II. Lanes J, K,and L represent λHtM4 DNA initially digested with *Bgl*II and then digested with *Kpn*I, *Hin*dIII or *Eco*RI. Lanes N and O contain λHtM4 DNA digested with *Eco*RI-*Kpn*I or *Eco*RI-*Hin*dIII. *Hin*dIII-*Kpn*I digested DNA is shown in lane P. Size marker DNAs are in lanes M where λ-DNA digested with *Bgl*II is in the left most lane and with *Hin*dIII in the right marker lane. (B) Radioautograph of a Southern transfer of the gel in (A) which had been hybridized to the *X. laevis* tRNA^{Tyr} gene probe. It is assumed that in each case where more than one hybridizing band is observed, the fastest moving band is the complete digestion product and all slower moving bands result from incomplete digestion. The second lane from the right was an unrelated experiment.

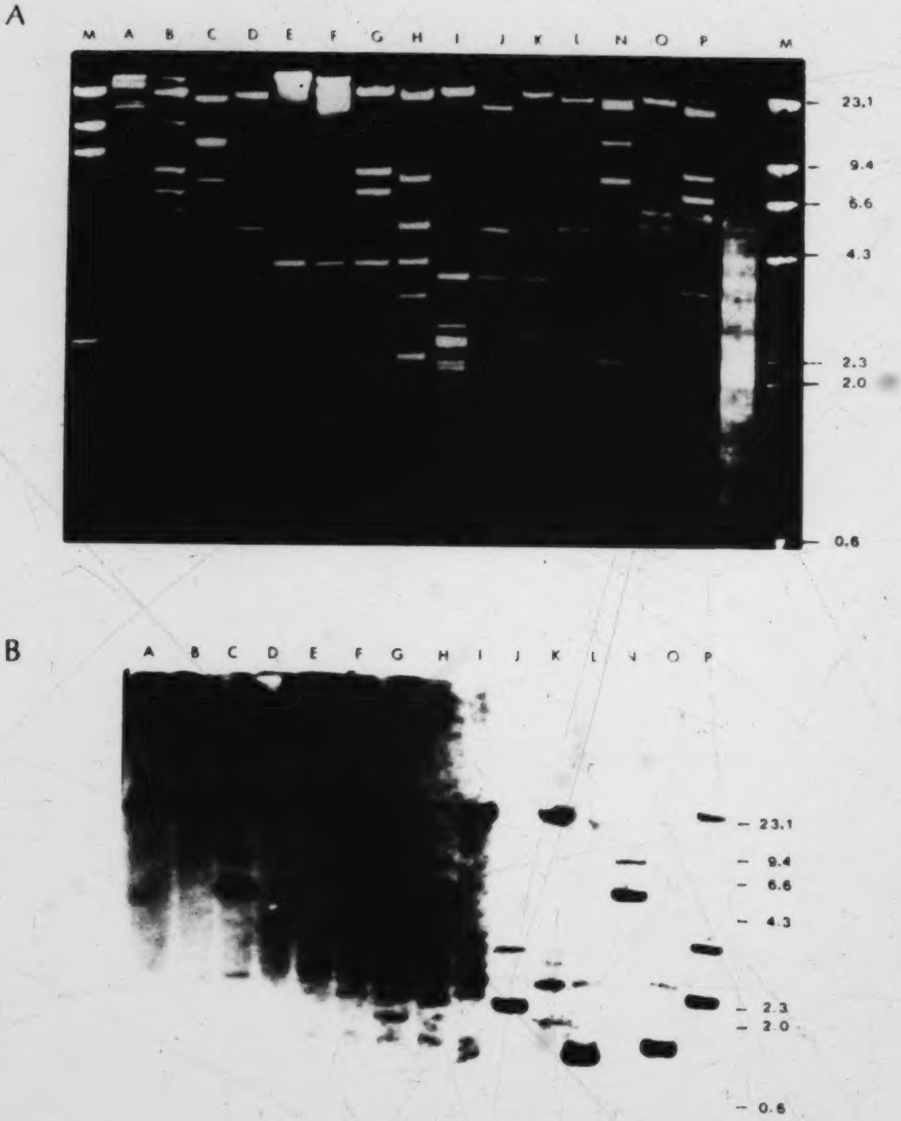
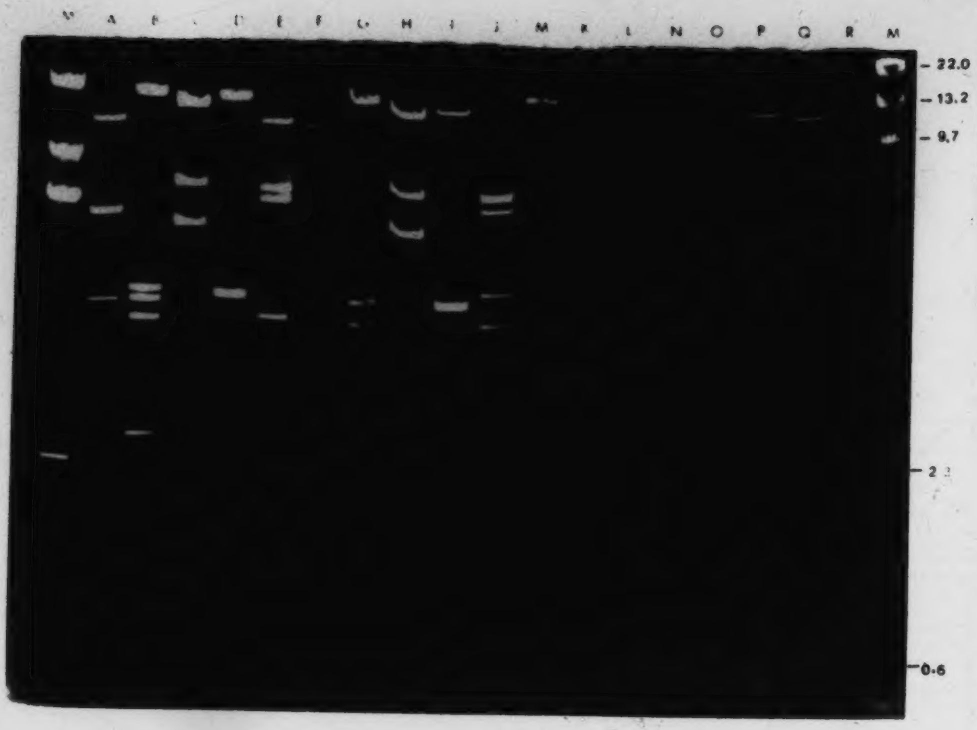

A
M A B C D E F G H I J K L N O P M
23.1
9.4
6.6
4.3
2.3
2.0
0.6
B
A B C D E F G H I J K L N O P
23.1
9.4
6.6
4.3
2.3
2.0
0.6

Figure 5. Restriction endonuclease digestion, Southern transfer and hybridization of λHtM6 DNA. Each digestion contained 2 µg of DNA. The generated DNA fragments were separated on a 0.75% agarose gel and were visualized by ethidium bromide-staining and UV-illumination. (A) Restriction enzyme digestion of λHtM6 DNA. Lanes M contain λ-DNA digested with *Bgl*II. Lanes A through E contain λHtM6 DNA digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII or *Mlu*I. *Xho*I-digested λHtM6 DNA is shown in lanes F through J, after further digestion with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, or *Mlu*I respectively. Lanes K, L, N and O are DNA doubly-digested with *Bam*HI-*Bgl*II, *Bam*HI-*Eco*RI, *Bam*HI-*Hin*dIII or *Bam*HI-*Mlu*I. *Hin*dIII-*Bgl*II digested DNA is in lane P. Lane Q contains λHtM6 DNA which was digested with *Hin*dIII and *Eco*RI. Lane R shows DNA digested with *Hin*dIII and *Mlu*I. (B) Radioautograph of a Southern transfer of the gel in (A) to a nitrocellulose membrane to which was hybridized the *X. laevis* $tRNA^{Tyr}$ gene probe. The variable number of hybridizing DNA fragments observed for different digestions results from multiple $tRNA^{Tyr}$ genes.

A


M A B C D E F G H I J M K L N O P Q R M
22.0
13.2
9.7
2.3
0.6

B

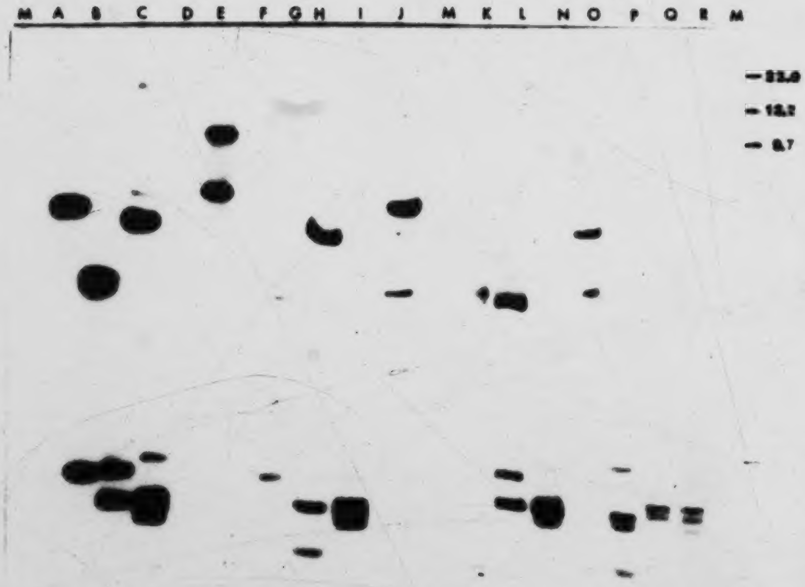

M A B C D E F G H I J M K L N O P Q R M
22.0
13.2
9.7

Table 1. Size estimation of DNA fragments from λHtM2 after restriction endonuclease digestion.

*Bam*HI	*Bgl*II	*Eco*RI	*Hin*dIII	*Kpn*I	*Mlu*I	*Xho*I
18.0* x2	21.0	19.9	20.5	17.4 x2	29.7*	-
5.6	9.6	16.0*	7.8	5.0*	9.8	-
3.9	4.8	11.0	5.7 x2	3.5	5.1	-
1.4	2.3	-	1.9	2.0	2.3	-
-	2.1	-	1.8*	1.5	-	-
-	1.6	-	1.4	-	-	-
-	1.4* x2	-	1.0	-	-	-
-	0.8	-	-	-	-	-
-	0.6	-	-	-	-	-
-	0.5	-	-	-	-	-

(1) These data have been compiled from several restriction mapping experiments.

(2) The numbers represent DNA fragment lengths in kb.

(3) * refers to DNA fragments which contained a tRNA gene.

Table 2. Size estimation of DNA fragments from λHtM4 after restriction endonuclease digestion.

BamHI	*BglI*	*EcoRI*	*HindIII*	*KpnI*	*MluI*	*XhoI*
36.1*	23.5*	19.9	22.5*	25.1*	27.5*	26.5*
5.5	4.8	11.0	8.2	17.4	9.8	21.0
3.9	3.8	7.5*	6.7	3.5	5.1	-
1.5	2.7	3.2	5.7	1.5	3.2	-
-	2.5	2.3 x2	2.2	-	2.3	-
-	2.4	1.5	1.3	-	-	-
-	2.1	-	0.9	-	-	-
-	1.3	-	-	-	-	-
-	1.0	-	-	-	-	-
-	0.9	-	-	-	-	-
-	0.6	-	-	-	-	-
-	0.5	-	-	-	-	-
-	0.4	-	-	-	-	-

(1) These data were compiled from several restriction mapping experiments.

(2) The numbers refer to DNA fragment lengths in kb.

(3) * refers to those DNA fragments which contained a tRNA gene.

Table 3. Size estimation of DNA fragments from λHtM6 after restriction endonuclease digestion.

*Bam*HI	*Bgl*II	*Eco*RI	*Hin*dIII	*Mlu*I	*Xho*I
17.8	23.3	19.9	21.5	17.5*	29.0*
9.2*	5.5*	11.0	5.7 x2	11.0*	11.5
5.5	5.2*	7.5*	2.3	9.8	5.9*
3.8	4.8	2.2*	2.0*	5.1	-
1.5	2.7	1.8*	1.5*	2.3	-
-	2.0	1.3	1.4*	-	-
-	1.8*	1.0	1.3*	-	-
-	1.4	-	1.2	-	-
-	0.9	-	1.0	-	-
-	0.6	-	0.9	-	-
-	0.4	-	0.6	-	-
-	-	-	0.3	-	-

(1) These data have been compiled from several restriction endonuclease mapping experiments.

(2) The numbers refer to DNA fragment lengths in kb.

(3) * refers to DNA fragments which contained a tRNA gene(s).

could be determined by the size variation between successive DNA fragments. The resulting fragments were separated on an agarose gel. These data were used to construct the physical map of λHtM6 as shown in Fig. 6. The four putative $tRNA^{Tyr}$-encoding DNA fragments are not arranged in tandem, but rather are spread throughout the 15.5-kb human DNA fragment of λHtM6. The restriction maps of the three human DNA fragments are shown in Fig. 6.

To demonstrate that the isolated recombinant DNAs contained tRNA genes, samples of each were digested with *Eco*RI and *Hin*dIII separately and the DNA fragments were fractionated electrophoretically on a 0.75% agarose gel (Fig. 7A). After separation the DNA fragments were transferred to a nylon membrane and hybridized with the 267-bp *X. laevis* $tRNA^{Tyr}$ gene probe (Fig. 7B). After removal of the probe DNA, the same nylon filter was rehybridized with unfractionated rabbit liver [5'-^{32}P] tRNA (Fig. 7C). The hybridization signal produced by the ^{32}P-labeled tRNA was poor. This was most likely due to inefficient labeling of the unfractionated tRNA. With λHtM6 digested with *Eco*RI, only the 7.5-kb fragment containing two of the four $tRNA^{Tyr}$ genes hybridized to the tRNA probe. The four *Hin*dIII DNA fragments, each carrying a single $tRNA^{Tyr}$ gene, failed to hybridize efficiently to the rabbit liver tRNA after repeated attempts. The end-labeled rabbit liver tRNA did not hybridize with the control λ-DNA. However, it appeared to hybridize to a limited extent with human DNA fragments which apparently did not have $tRNA^{Tyr}$ genes.

Figure 6. Restriction endonuclease digestion maps of three recombinant bacteriophage DNAs. L END represents the left arm of the λ-Charon-4A phage vector. R END depicts the right arm of the vector. E is *Eco*RI, K is *Kpn*I, Bg is *Bgl*II, H is *Hin*dIII, Ba is *Bam*HI, M is *Mlu*I, S is *Sma*I, Xb is *Xba*I and Xh is *Xho*I. The dark rectangles indicate the DNA fragments which were subcloned into plasmid vectors for further characterization of the DNA segments containing the human tRNA genes.

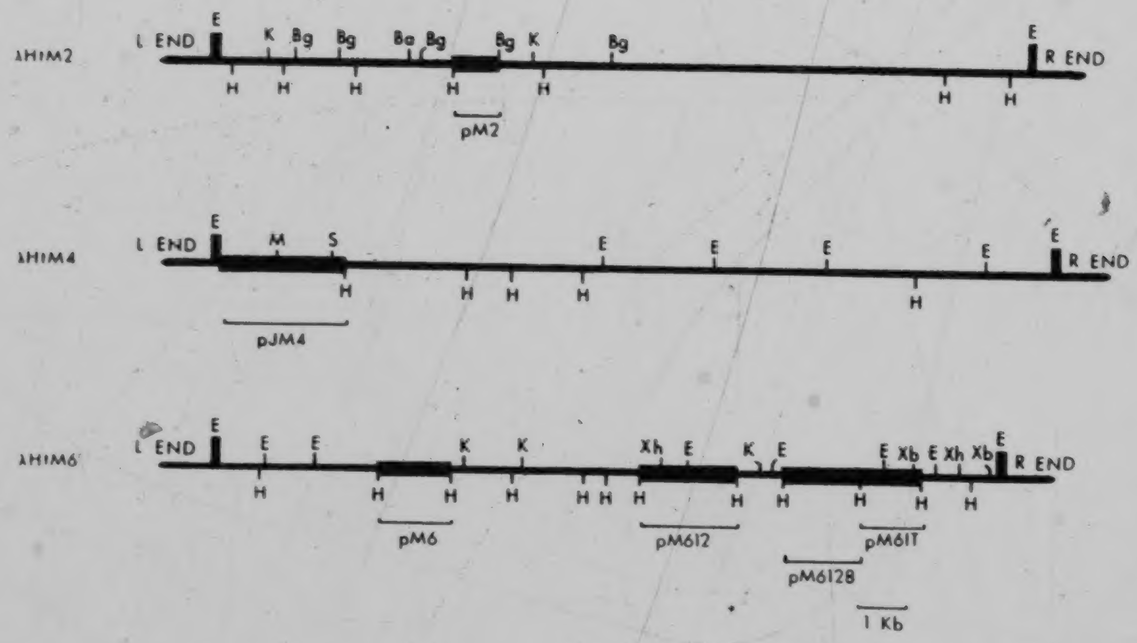
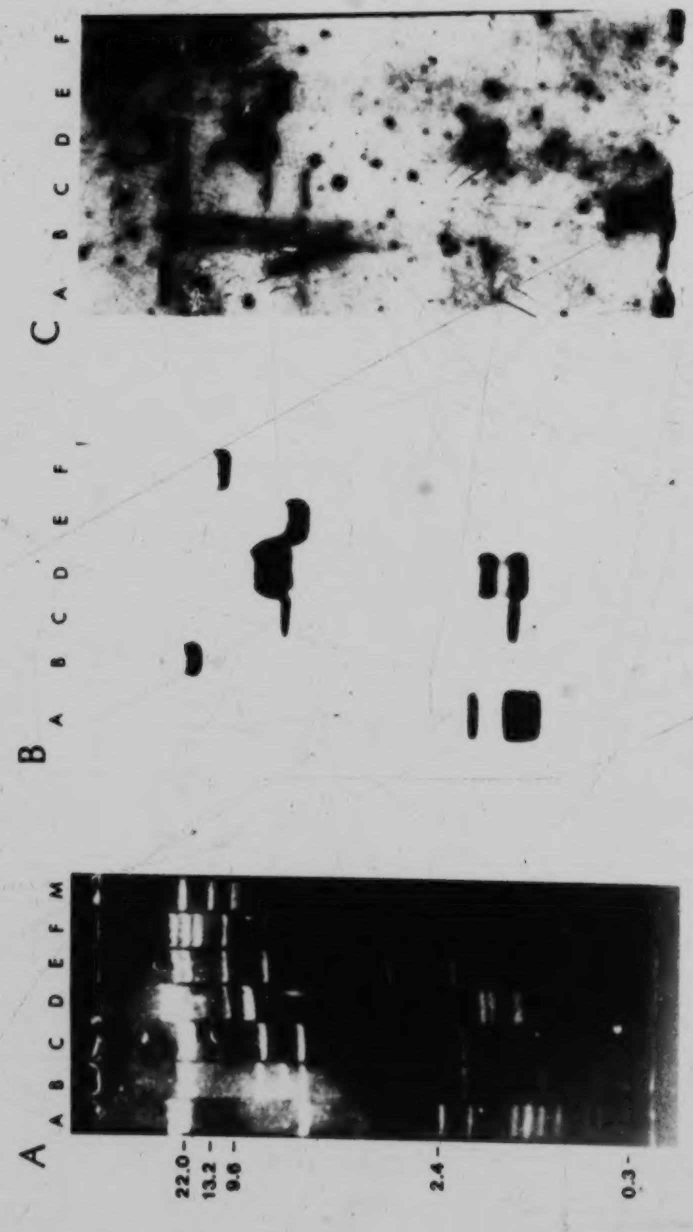

λHIM2
L END
E
K
Bg
Bg
Ba
Bg
Bg
K
Bg
E
R END
H
H
H
H
H
H
H
pM2
λHIM4
L END
E
M
S
E
E
E
E
E
R END
H
H
H
H
H
pJM4
λHIM6
L END
E
E
E
K
K
Xh
E
K
E
E
Xb
E
Xh
Xb
E
R END
H
H
H
H
H
H
H
H
H
H
H
H
pM6
pM612
pM6128
pM61T
1 Kb

Figure 7. Hybridization of unfractionated rabbit liver tRNA and a tRNA^{Tyr} gene probe to three recombinant bacteriophage DNAs. (A). Ethidium bromide-stained gel showing λHtM6, λHtM4 and λHtM2 DNA digested with*Hin*dIII (lanes A, B and C). Lanes D, E and F show λHtM6, λHtM4 and λHtM2 DNAs digested with *Eco*RI. The DNA size marker lane (M) shows λ-DNA which had been digested with *Bgl*II. (B). Radioautograph of a Southern transfer of the gel illustrated in (A) to which the ^{32}P-labeled *X. laevis* tRNA^{Tyr} gene probe was hybridized. (C) The same Southern transfer as in (B) except that the unfractionated ^{32}P-labeled tRNA probe had been hybridized after removal of the previous probe.


A
A B C D E F M
22.0
13.2
9.6
2.4
0.3
B
A B C D E F
C
A B C D E F

(c) Construction of recombinant plasmid subclones encoding human tRNAs

To characterize the isolated human $tRNA^{Tyr}$ genes further, sub-fragments from each bacteriophage clone were inserted into plasmid vectors. Since the possibility existed that the bacteriophage clones could contain more than one tRNA gene, DNA fragments containing the individual tRNA genes had to be isolated so that each tRNA gene could be investigated independently. In general, recombinant pAT153 subclones were generated by shotgun-cloning the fragments from a complete digest of the recombinant bacteriophage DNA into pAT153 DNA cleaved with the appropriate restriction enzyme(s). The human DNA fragment was subcloned into a restriction site (or sites) directly in front of the tetracycline resistance gene of pAT153. Those colonies displaying an AP^r, TC^s phenotype were screened for the presence of human tRNA genes by colony hybridization (Grunstein and Hogness, 1975). Those bacteria which contained recombinant plasmids and displayed hybridization to the *X. laevis* $tRNA^{Tyr}$ gene probe were isolated.

Five recombinant plasmids were constructed. A 2.5-kb *Eco*RI-*Hin*dIII DNA fragment from λHtM4 was inserted into pAT153 to construct the plasmid pJM4 about 6.1-kb. The four tRNA-encoding *Hin*dIII fragments from λHtM6 were subcloned into pAT153 and comprised the pM6 series of recombinant plasmids. The 2.0-kb *Hin*dIII fragment was subcloned, along with a 0.35-kb *Hin*dIII fragment to form pM612 (6-kb). The 1.5-kb *Hin*dIII fragment was subcloned to generate the recombinant plasmid pM6128 (5.1-kb). The plasmid pM6 was constructed by inserting the 1.4-kb *Hin*dIII fragment from λHtM6 to generate a plasmid of 5.0-kb. The 1.3-kb *Hin*dIII fragment was subcloned to generate the recombinant plasmid pM6IT, which was 9.2-kb. This size resulted from the ligation of several additional *Hin*dIII fragments from λHtM6, into a single plasmid. Further experiments (data not shown) indicated that only the 1.3-kb *Hin*dIII fragment contained tDNA. Since only the 1.3-kb *Hin*dIII fragment encoded a human tRNA^{Tyr} it could be independently characterized.

(d) Physical mapping of pJM4

A 2.5-kb *Eco*RI-*Hin*dIII DNA fragment from λHtM4 was subcloned into pAT153 also digested with *Eco*RI and *Hin*dIII as described in MATERIALS AND METHODS. A limited physical map of pJM4 was constructed. The physical map shown in Fig. 8 was constructed by methods previously described. The human tRNA gene is located 400-bp from the *Eco* RI site of the human DNA fragment.

(e) Physical maps of pM6, pM6128, pM612 and pM6IT

The recombinant bacteriophage λHtM6 when digested with *Hin*dIII, gave thirteen separate fragments, four of which contained tRNA^{Tyr} genes. Two of these DNA fragments were subcloned as described above. The plasmids pM6 and pM6128 contain *Hin*dIII fragments of 1.4-kb and 1.5-kb, respectively, from λHtM6 cloned into the *Hin*dIII site of pAT153. The physical maps of both plasmids were determined by standard restriction mapping procedures (Maniatis *et al.*, 1982; Smith and Birnsteil, 1976).

Figure 8. The restriction endonuclease map of pJM4. The 2.5-kb *Eco*RI-*Hin*dIII DNA fragment from λHtM4 was subcloned into the plasmid vector pAT153. The dark circle depicts the plasmid vector DNA in which AP represents the ampicillin resistance gene and TC denotes the tetracycline resistance gene of pAT153. The following abbreviations indicate the restriction sites of the enzymes in parenthesis; E (*Eco*RI), Hd (*Hin*dIII), Pv (*Pvu*II), Sm (*Sma*I), Hc (*Hin*cII), Ps (*Pst*I) and Sp (*Sph*I). The open box represents the tRNA gene and the arrow above it indicates the direction of transcription. The single-headed arrows indicate the DNA fragments sequenced. The boxed 'H's denote a *Hae*III fragment used in sequencing the gene.

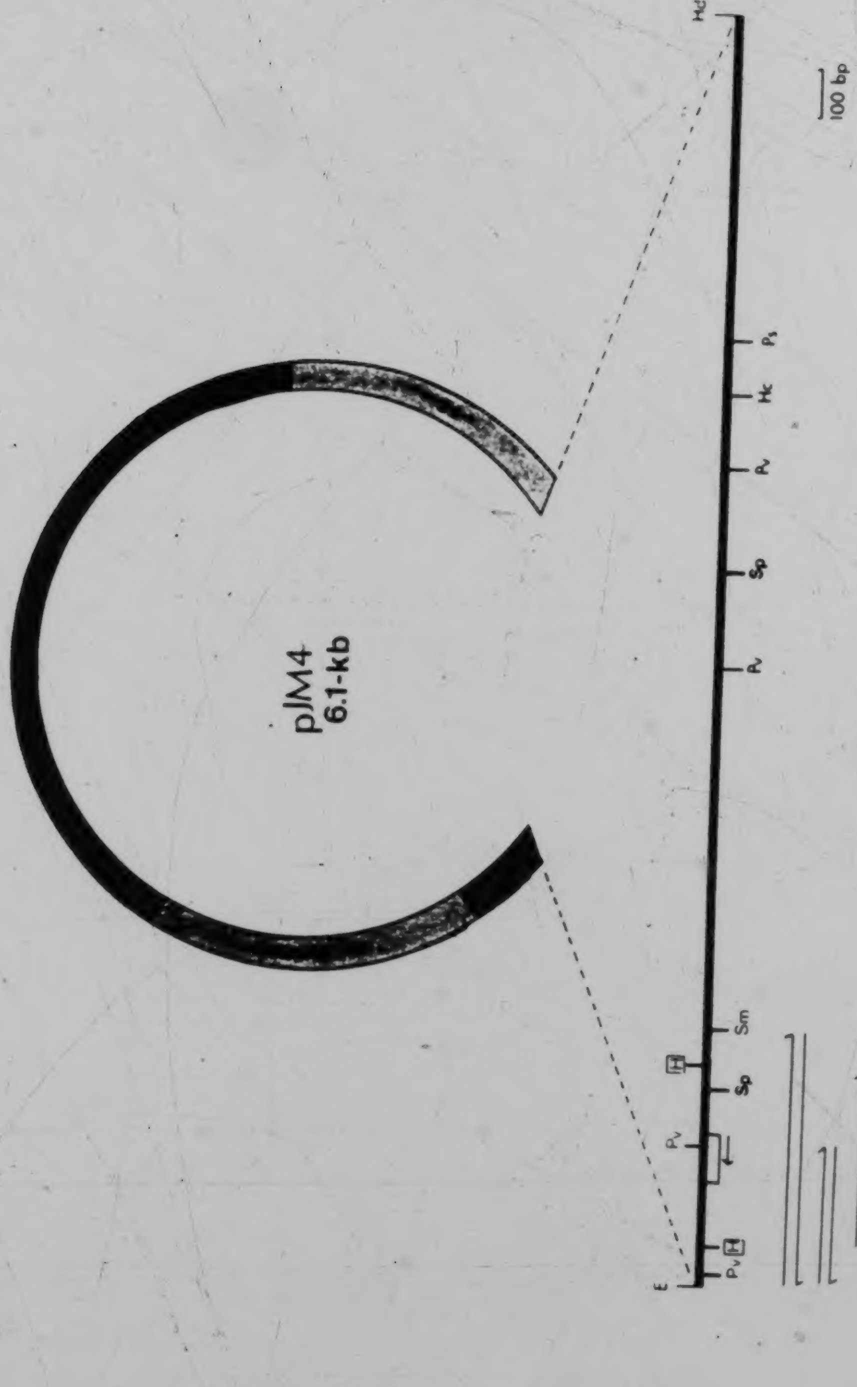

pJM4
6.1-kb
Hd
Ps
Hc
Pv
Sp
Pv
Sm
H
Sp
Pv
H
Pv
E
100 bp

Figure 9 represents the physical map of pM6. The human tRNA^{Tyr} gene occurs within a 280-bp *Sau*3AI fragment, and this complete fragment was sequenced. The restriction map of the recombinant plasmid pM6128 is shown in Fig.10. The tRNA gene is located near the left end of the human DNA insert, and is contained within a 350-bp *Sau*3AI fragment.

The 2.0-kb *Hin*dIII fragment from λHtM6 was subcloned into pAT153 along with a 0.35-kb *Hin*dIII fragment and this recombinant was designated pM612. The recombinant plasmid pM6IT encoded the final tRNA^{Tyr} from λHtM6. The 1.3-kb DNA fragment was not independently subcloned into pAT153. Instead, pM6IT contained five *Hin*dIII DNA fragments derived from λHtM6 of 2.3-kb, 1.3-kb, 1.0-kb, 0.65-kb and 0.35-kb. This particular plasmid was 9.2-kb. Since only the 1.3-kb DNA fragment encoded a human tRNA, it was used to analyse the coding sequence of the fourth human tRNA gene of λHtM6.

Several restriction endonuclease sites in both pM612 and pM6IT were located by single- and double-digestions with six-base specific restriction enzymes. As with the other recombinant plasmids, the locations of the restriction sites were determined by partial digestion mapping of end-labeled plasmid DNA. An example of these data are presented for pM612 which was digested with *Bam*HI (position 375 on the pAT153 map) and the DNA labeled as described above (Fig. 11). The enzyme *Sal*I released a 276-bp fragment (position 651 on the pAT153 map) and left a unique radiolabeled terminus to serve as a reference point to order the location of the other sites. The physical maps of pM612 and pM6IT are shown in Fig. 12 and Fig. 13 respectively.

Figure 9. The restriction endonuclease map of pM6. The dark circle depicts the plasmid DNA in which AP denotes the ampicillin resistance gene and TC represents the tetracycline resistance gene of pAT153. The open box indicates the tRNA gene and the arrow above it denotes the direction of transcription. The single barbed arrows represent the overlapping *Hae*III and *Sau*3AI fragments sequenced to determine the tRNA gene sequence. Hd is *Hin*dIII, A is *Alu* I, H is *Hae* III and S is *Sau*3AI.

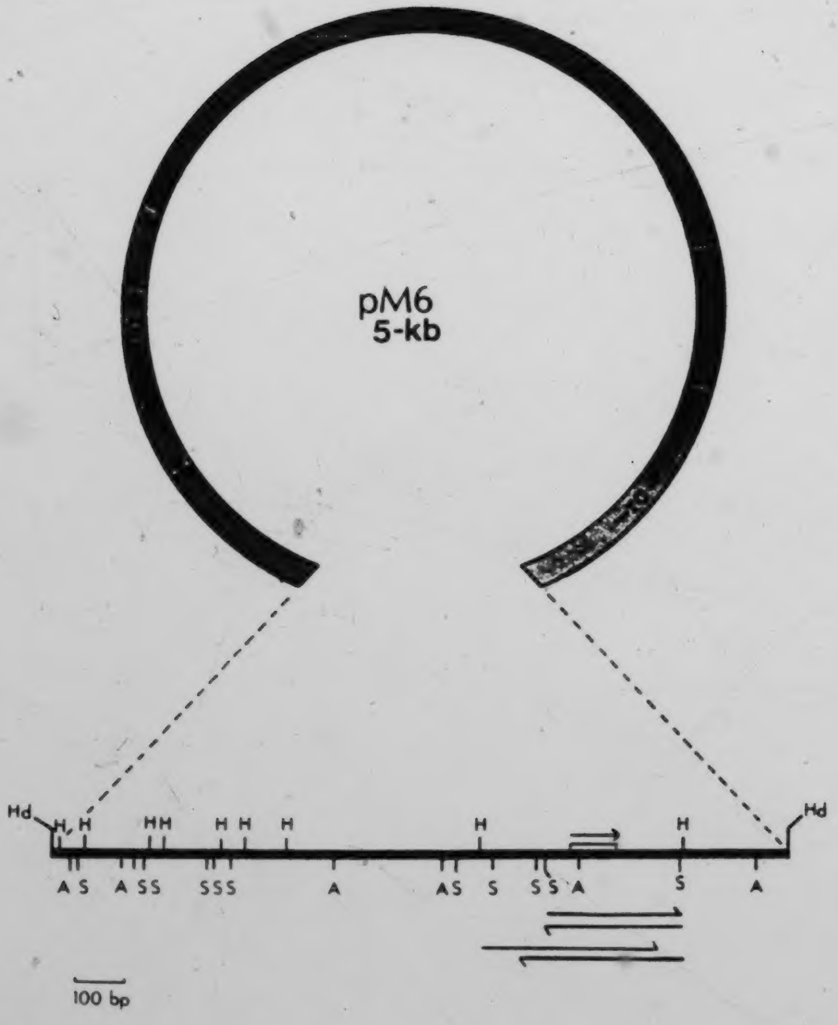

pM6
5-kb
Hd
H
H
HH
H
H
H
H
H
Hd
A
S
A
SS
SSS
A
AS
S
SS
A
S
A
100 bp

Figure 10. The restriction endonuclease map of pM6128. The dark circle represents the plasmid DNA in which AP denotes the ampicillin resistance gene and TC represents the tetracycline resistance gene contained within pAT153. The open box depicts the tRNA gene and the arrow immediately below the open box indicates the direction of transcription of this gene. The single headed arrows show the overlapping *Hae*III and *Sau*3AI fragments which were used to sequence the tRNA gene within this recombinant plasmid. Hd is *Hin*dIII, H is *Hae*III, A is *Alu*I and S is *Sau*3AI.

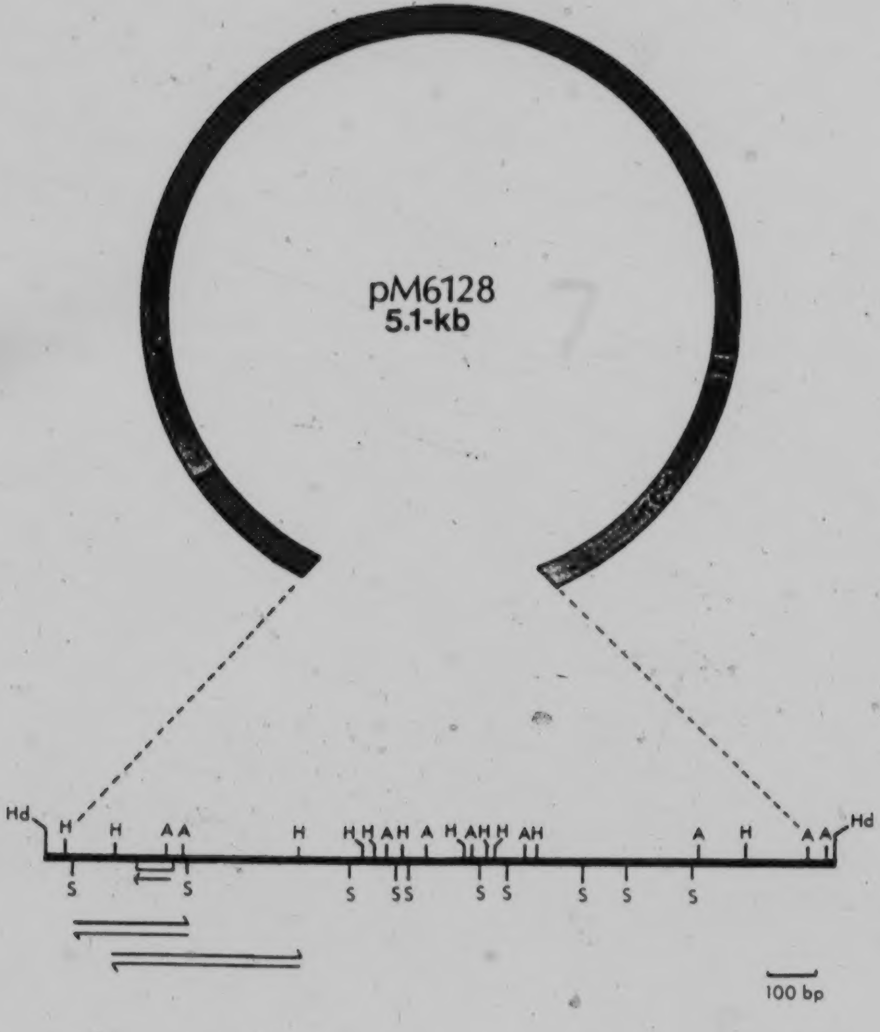

pM6128
5.1-kb
Hd
H
H
A
A
H
H
H
A
H
A
H
A
H
H
A
H
A
H
A
A
Hd
S
S
S
S
S
S
S
S
S
S
100 bp

Figure 11. Radioautograph of partially digested ^{32}P-labeled pM612 DNA. The recombinant plasmid pM612 was labeled and digested as previously described. The partially digested DNA fragments were separated on a 0.75% agarose gel, precipitated directly in the gel with 7% TCA, and the gel was dried as described. The letters at the top of the Figure, A, B, and C, represent 0.5 u, 1.0 u and 3 u of the indicated restriction endonuclease, respectively. The size markers were generated by digesting λ-DNA with *Cla*I.

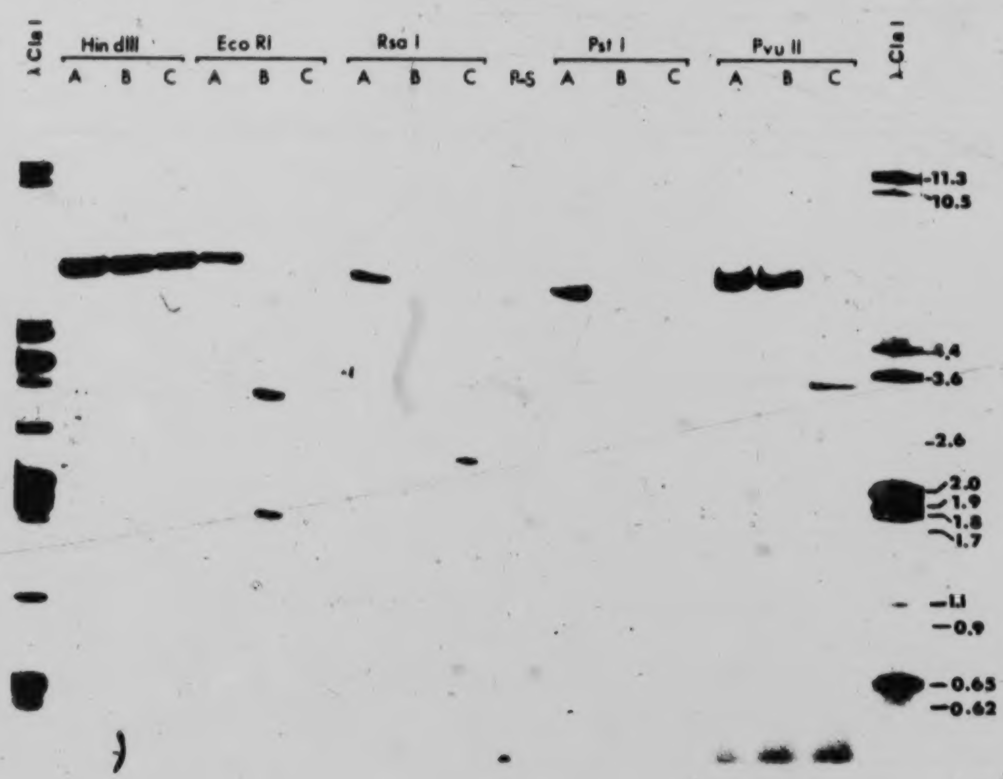

λ Cla I
Hin dIII
A B C
Eco RI
A B C
Rsa I
A B C
R-S
Pst I
A B C
Pvu II
A B C
λ-Cla I
11.3
10.5
4.4
3.6
2.6
2.0
1.9
1.8
1.7
1.1
0.9
0.65
0.62

Figure 12. The restriction endonuclease map of pM612. A 2.0-kb *Hin*dIII fragment from λHtM6 was subcloned into the plasmid vector pAT153. The dark circle represents the plasmid DNA; AP indicates the ampicillin resistance gene and TC denotes the tetracycline resistance gene. The open box represents the tRNA gene and the arrow above it indicates the direction of gene transcription. The single barbed arrows show overlapping DNA fragments which were used to sequence the human tRNA gene. The boxed 'H's refer to an 800-bp *Hae*III DNA fragment which was only partially sequenced due to its extended length. No other *Hae*III restriction enzyme sites were placed on the restriction map. Hd is *Hin*dIII, R is *Rsa*I, Ps is *Pst*I, Pv is *Pvu*II, Ec is *Eco*RI, Sa is *Sal*I and Sm is *Sma*I.

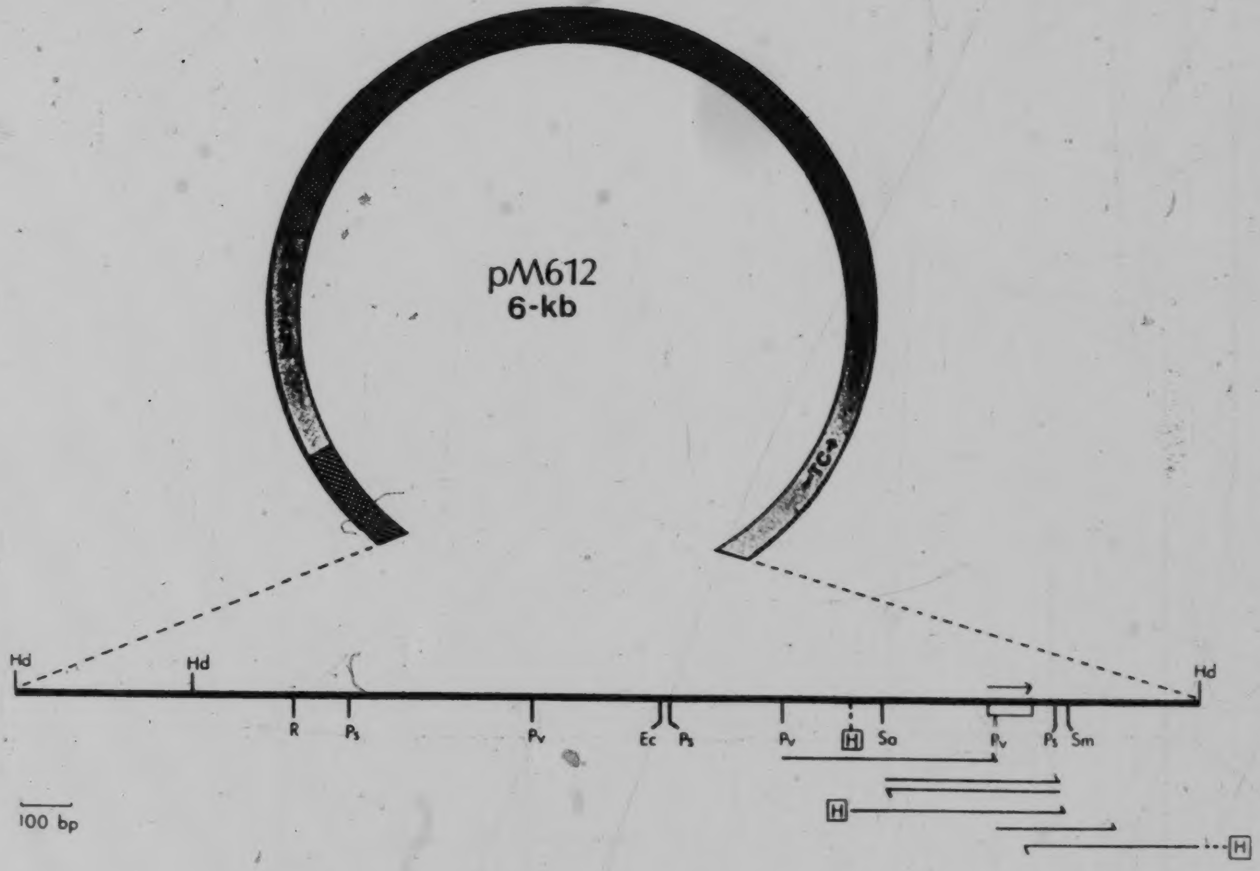
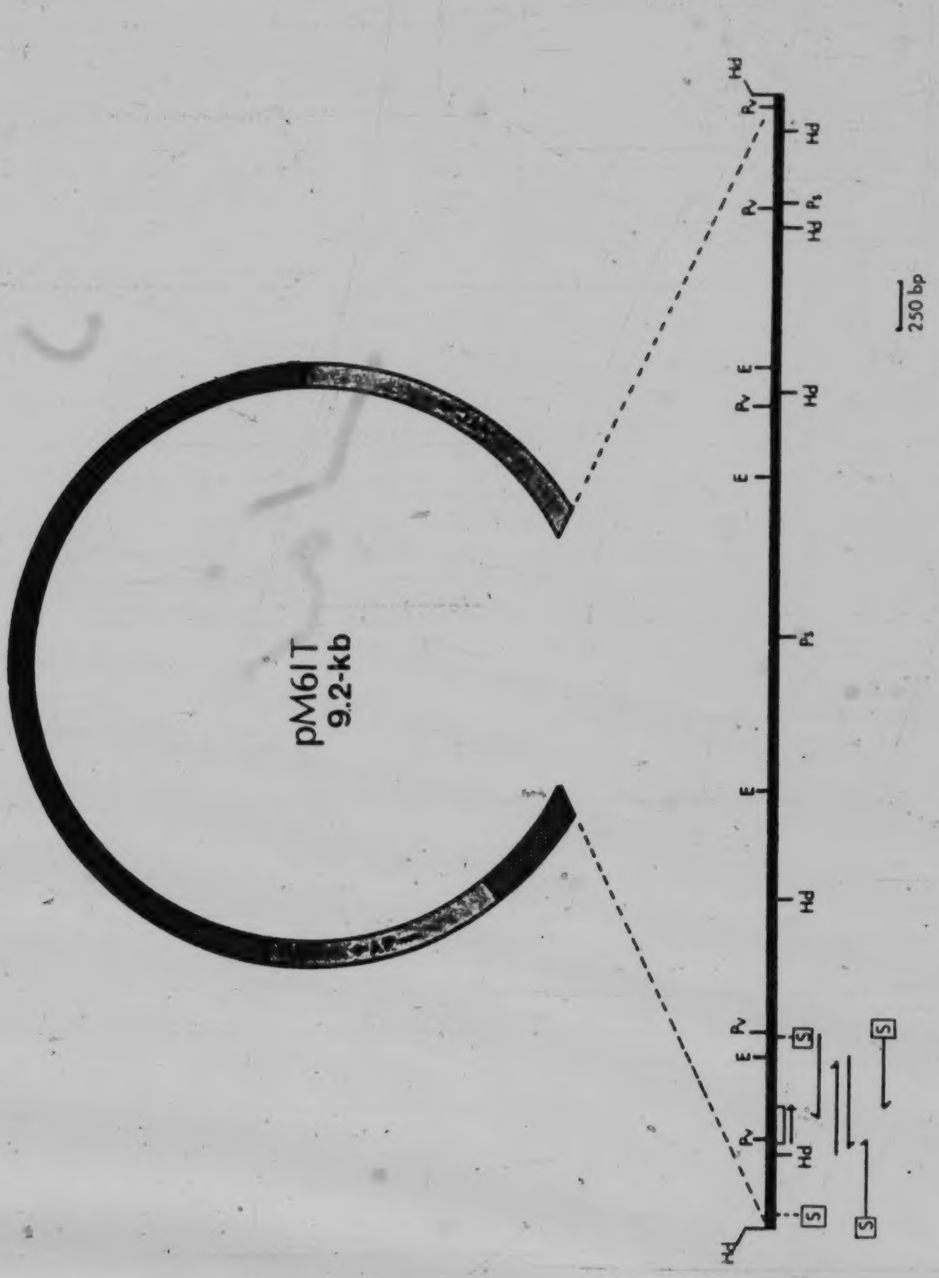

pM612
6-kb
TC
Hd
Hd
R
Ps
Pv
Ec
Ps
Pv
H
Sa
Pv
Ps
Sm
Hd
H
H
100 bp

Figure 13. The restriction endonuclease map of pM6IT. A 1.3-kb *Hin*dIII fragment from λHtM6 was subcloned into the plasmid vector pAT153, along with other *Hin*dIII fragments from the recombinant bacteriophage (see text). The plasmid DNA is represented by the dark circle where AP indicates the ampicillin resistance gene and TC denotes the tetracycline gene. Hd is *Hin*dIII, Pv is *Pvu*II, E is *Eco*RI, Ps is *Pst*I. The boxed 'S's indicate a large *Sau*3AI fragment which was only partially sequenced. No other *Sau*3AI restriction sites were placed on the map. The open box represents the human $tRNA^{Tyr}$ gene and the arrow immediately under the box indicates the direction of gene transcription. The single barbed arrows indicate the DNA fragments which were used to determine the nucleotide sequence of the human tRNA gene within this recombinant plasmid.


pM61T
9.2-kb
Hd
Pv
E
Ps
S
Ap
250 bp

The five recombinant plasmids described above each contained a single human tRNA gene as determined by hybridization to the *X. laevis* tRNA^{Tyr} gene probe. However, there remained the possibility that these plasmids simply had DNA sequences which cross-hybridized to the tRNA^{Tyr} gene probe DNA, as for example, tRNA pseudogenes or repetitive DNA sequences. To ascertain the nature of these hybridizing DNA fragments and to determine the species of tRNA genes which may have been present, the DNA sequence spanning each putative tRNA^{Tyr} gene was determined.

(f) Nucleotide sequence and *in vitro* expression of five human tRNA^{Tyr} genes

Small DNA fragments which included the human tRNA genes from each recombinant plasmid DNA were cloned into either M13mp10, M13mp11, M13mp18 or M13mp19 (Yanisch-Perron *et al.*, 1985). These clones were sequenced by the dideoxynucleoside triphosphate chain termination procedure as described by Sanger *et al.* (1977). Overlapping complementary sequences were generally isolated and compared to determine the validity of the generated DNA sequence.

Overlapping *Hae*III- and *Sau*3AI-fragments from both pM6 and pM6128 were inserted into the *Sma*I and *Bam*HI sites, respectively, of either M13mp10 or M13mp11. These were sequenced as described. Sequence analysis of the cloned DNA fragments revealed that each plasmid encoded a human tRNA^{Tyr} as indicated by the anticodon sequence GTA. Both tRNA^{Tyr} gene-coding regions were essentially identical to the *X. laevis* tRNA^{Tyr} gene (Müller and Clarkson, 1981).

The gene encoded by pM6 differed from the *X. laevis* $tRNA^{Tyr}$ gene by a G to A transition at position 57. This difference resulted in the loss of *Hin*fI and *Taq*I sites in the 3'-end of the gene sequence. The 3'-CCA terminus is not encoded by either gene. The sequence spanning the human $tRNA^{Tyr}$ gene in the recombinant plasmid pM6 is shown in Fig. 14. The sequence spanning the human $tRNA^{Tyr}$ gene contained within the recombinant plasmid pM6128 is illustrated in Fig. 15. Beyond both $tRNA^{Tyr}$ genes is a short T tract located 13-bp downstream from the 3'-end of the genes. These are the suggested transcription termination signals for RNA polymerase III (Bogenhagen and Brown, 1981). There is also considerable homology between the 3'-flanking regions of these two genes and the termination signals.

The most striking and predicted feature of these genes is the presence of a 21-bp intervening sequence which occurs one base to the 3'-side of the anticodon. Both intervening sequences begin with the same nucleotide A, although, as will be shown, not all the human $tRNA^{Tyr}$ genes have introns which start with the same sequence. The intervening sequences end with the same sequence, GAC. The homology between the two introns is not complete; only 14 of the 21-bp are conserved.

Considerable homology is present between the 5'-flanking regions of the $tRNA^{Tyr}$ genes in pM6 and pM6128 (will be shown in Fig. 19). Within the first 60 bases immediately preceding the genes there are four regions, -57 to -36, -34 to -22, -20 to -16 and -6 to -2, with complete homology. A short 10-bp sequence located within the $tRNA^{Tyr}$ gene intron (pM6128) at 48 to 57 is identical to

Figure 14. Nucleotide sequence of the tRNA^{Tyr} gene within pM6. The upper strand of DNA represents the RNA-like (non-coding) strand of DNA. The tRNA gene is 73 nt in length and the 3'-CCA terminus of the mature tRNA is not encoded by the DNA sequence. The tRNA gene is boxed and starts at position 75. The dots under the sequence are the regions of homology discussed in the text. This gene is interrupted by a 21-bp intervening sequence. A short tract of T residues is overlined and is the suggested transcription termination signal for RNA polymerase III.

```
        10         20         30         40         50         60
GATCTCCGGT GGTCCAGGGA CTTGGCTTCC TCCATTTGCA GAAAGTCCAG TGACCCAGCC
CTAGAGGCCA CCAGGTCCCT GAACCGAAGG AGGTAAACGT CTTTCAGGTC ACTGGGTCGG

        70         80         90        100        110        120
TTAACAGTGT GCATCCTTCG ATAGCTCAGC TGGTAGAGCG GAGGACTGTA GACTGCGGAA
AATTGTCACA CGTAGGAAGC TATCGAGTCG ACCATCTCGC CTCCTGACAT CTGACGCCTT

       130        140        150        160        170        180
ACGTTTGTGG ACATCCTTAG GTCGCTGGTT CAATTCCGGC TCGAAGGAAG CGCCTGACTC
TGCAAACACC TGTAGGAATC CAGCGACCAA GTTAAGGCCG AGCTTCCTTC GCGGACTGAG

       190        200        210        220        230        240
TTTTGCGCAC AATGCTGCCT GGCTGCACCT GTTCCTCGTC AAAGACCTTG CAGCCTTCCA
AAAACGCGTG TTACGACGGA CCGACGTGGA CAAGGAGCAG TTTCTGGAAC GTCGGAAGGT

       250        260        270        280        290
GTCATAACTA CACTTTCCCC AGGAAAACCC AGCAAAATCC TGCCTTTCCT GATC
CAGTATTGAT GTGAAAGGGG TCCTTTTGGG TCGTTTTAGG ACGGAAAGGA CTAG
```

Figure 15. Nucleotide sequence of the $tRNA^{Tyr}$ gene within pM6128. The upper strand of DNA represents the non-coding strand of DNA. The $tRNA^{Tyr}$ gene is boxed and is interrupted by a 21-bp intron. The gene starts at position 231. The dots under the sequence indicate regions of homology within the intervening sequence and the 5'-flanking region. A short T tract in the 3'-flanking region is overlined and is the suggested transcription termination signal for RNA polymerase III.

```
        10         20         30         40         50         60
CCGCAGGCGA GACGTCCCGT CCTGGATTGT GGCTATCAGC GCTCTGGGAC GCGACCAAAC
GGCGTCCGCT CTGCAGGGCA GGACCTAACA CCGATAGTCG CGAGACCCTG CGCTGGTTTG

        70         80         90        100        110        120
CACAGTCGGA GGATTTGCTC CTCACCTGAG AGGTGCGCGG TGGCAACCAG CGCAAGGTTC
GTGTCAGCCT CCTAAACGAG GAGTGGACTC TCCACGCGCC ACCGTTGGTC GCGTTCCAAG

       130        140        150        160        170        180
TCTTCTAAGG CGGGTTCCAA TCAACTCTAA GTGTGTTGAC TCCAGCGTTC CAAGGACTTG
AGAAGATTCC GCCCAAGGTT AGTTGAGATT CACACAACTG AGGTCGCAAG GTTCCTGAAC

       190        200        210        220        230        240
GCTTCCTCCA TTTGCGGAAA GTCCAGTGAT CCAGCTCTTG CAGCGTGCAC CCTTCGATAG
CGAAGGAGGT AAACGCCTTT CAGGTCACTA GGTCGAGAAC GTCGCACGTG GGAAGCTATC
        .. ........

       250        260        270        280        290        300
CTCAGCTGGT AGAGCGGAGG ACTGTAGATT GTACAGACAT TTGCGGACAT CCTTAGGTCG
GAGTCGACCA TCTCGCCTCC TGACATCTAA CATGTCTGTA AACGCCTGTA GGAATCCAGC
                                        ...  .......

       310        320        330        340        350        360
CTGGTTCGAT TCCGGCTCGA AGGAAGTGCC CGATGCTTTT GCATGCAATG CCACCTGGTG
GACCAAGCTA AGGCCGAGCT TCCTTCACGG GCTACGAAAA CGTACGTTAC GGTGGACCAC

       370        380        390        400        410        420
CTGGTCAAAG GCCCTGCAGC CTCCAACTAG TATCCACCCA CACCCTCCCA GTCAAAACCC
GACCAGTTTC CGGGACGTCG GAGGTTGATC ATAGGTGGGT GTGGGAGGGT CAGTTTTGGG

       430        440        450        460
AGAGAAACCT TTCCTGATCA CCTGGTTTCC ACACCTGTGC TGGG
TCTCTTTGGA AAGGACTAGT GGACCAAAGG TGTGGACACG ACCC
```

position -42 to -33 of this gene's 5'-flanking sequence. A similar sequence occurs in the other tRNA^{Tyr} gene (pM6), however there is not as extensive a duplication of sequence, as only 7 of the 10-bp are identical.

The recombinant plasmids pM612 and pM6IT contain the remaining two genes from the tRNA gene cluster described above. These recombinant plasmids each have a human tRNA^{Tyr} gene similar to the two genes already described. Several overlapping DNA fragments were sequenced to determine that the gene within pM6IT was a human tRNA^{Tyr} gene, as indicated by the anticodon sequence GTA. The gene coding sequence (Fig. 16) is identical with that of the gene within pM6128. This gene (pM6IT) was 73-bp in length as were the other two genes. The intervening sequence of this gene is similar to the gene within pM6128. This gene also contains a short sequence within the intron (position 120 to 127; Fig 16) which is almost identical to an eight bp sequence in the 5'-flanking region (position 67 to 74; Fig 16). The recombinant plasmid pM612 also contains a human tRNA^{Tyr} gene (Fig. 17). The coding sequence is identical to the gene encoded within pM6128, with the notable exception of a conservative G to A transition at position 63 (position 308; Fig. 17) of the mature tRNA. This change would slightly increase the stability of the stem structure formed in this region of the molecule. The gene (pM612) is also interrupted by a 21-bp intervening sequence as were the other three genes and the intron has in common the 3'-terminal GAC. In fact, the intron is almost identical with that found in pM6128, having a T at the seventh position of the intron instead of a C. Located within the intron

Figure 16. Nucleotide sequence of the tRNA^{Tyr} gene within pM6IT. The upper strand of DNA depicts to the non-coding strand of DNA. The tRNA^{Tyr} gene is boxed and starts at position 79. The dots under the sequence indicate regions of homology within the 5'-flanking region and the intron. The suggested transcription termination signal is a short tract of T residues and is overlined.

```
        10         20         30         40         50         60
AAGCTTATTG GACGACTAGA GTTGCAAGGA GTCTTGCATT TGCTGAAAGA TCAATGACCC
TTCGAATAAC CTGCTGATCT CAACGTTCCT CAGAACGTAA ACGACTTTCT AGTTACTGGG

        70         80         90        100        110        120
AACCCCAGAA ACGTGCGCCC TTCGATAGCT CAGCTGGTAG ACGGAGGACT GTAGCCTGTA
TTGGGGTCTT TGCACGCGGG AAGCTATCGA GTCGACCATC TGCCTCCTGA CATCGGACAT

       130        140        150        160        170        180
GAAACATTTG TGGACATCCT TAGGTCGCTG GTTCGATTCC GGCTCGAAGG AGCTGCCGTA
CTTTGTAAAC ACCTGTAGGA ATCCAGCGAC CAAGCTAAGG CCGAGCTTCC TCGACGGCAT

       190        200        210        220        230        240
TTCTTTTGCA CACGCACGCA CCAAAACTAC GTGGCTGCAT CTCTGCCTGG TCAAAGGCTT
AAGAAAACGT GTGCGTGCGT GGTTTTGATG CACCGACGTA GAGACGGACC AGTTTCCGAA

       250        260        270        280        290        300
TGCAGCCAGC ATCCACACTC TCCCAGGAGA AACCTAGCAA GGCCTTTCCG GATTACCCAG
ACGTCGGTCG TAGGTGTGAG AGGGTCCTCT TTGGATCGTT CCGGAAAGGC CTAATGGGTC

       310        320        330        340        350        360
CTTACCCACA GCCTATGCTG TGCGCTTGGG AGTCTGCTCA TTCTTCAAGT CATTGCTTTC
GAATGGGTGT CGGATACGAC ACGCGAACCC TCAGACGAGT AAGAAGTTCA GTAACGAAAG

       370        380        390        400        410        420
TCTACTATCT TCAAATTTTT TATATAATCT GGGATTTTTA TCTCCAAGTC ATTGATTTTT
AGATGATAGA AGTTTAAAAA ATATATTAGA CCCTAAAAAT AGAGGTTCAG TAACTAAAAA

       430        440        450        460        470        480
ATGGGCCATT AGGTGACACT ATAACTGATA TTCTTATGAA ACTAGATGAA ATATTTGAAT
TACCCGGTAA TCCACTGTGA TATTGACTAT AAGAATACTT TGATCTACTT TATAAACTTA
```

Figure 17. Nucleotide sequence of the tRNA^{Tyr} gene within pM612. The upper strand of DNA depicts the non-coding strand of DNA. The tRNA gene is boxed and starts at position 225. The dots under the sequence indicate regions of homology within the intervening sequence and the 5'-flanking region of this gene. The overlined sequence is a short T tract which is the suggested transcription termination site for RNA polymerase III.

```
         10         20         30         40         50         60
GAGCTCCTCC TCGTGGATGG TGGCTATCAG AGCCCGAGAG ACACCAGGCA TCCTCGCCCA
CTCGAGGAGG AGCACCTACC ACCGATAGTC TCGGGCTCTC TGTGGTCCGT AGGAGCGGGT

         70         80         90        100        110        120
GAGGATTTGC TCCGCCCTGA AAGGGGTGTG GTGTCAAGCG GCGCAGGGTT CTCTTCCAAG
CTCCTAAACG AGGCGGGACT TTCCCCACAC CACAGTTCGC CGCGTCCCAA GAGAAGGTTC

        130        140        150        160        170        180
GTGGGTGGCC AACCAACCCA ACGCGTATTG GACCTCAAGC ATTCCAGGGA TGTGGCTCCC
CACCCACCGG TTGGTTGGGT TGCGCATAAC CTGGAGTTCG TAAGGTCCCT ACACCGAGGG

        190        200        210        220        230        240
TCTGTTTGCA GAAAGTCCAA TGAACCAGCT TTGATAGCAT GCATCCTTCG ATAGCTCAGC
AGACAAACGT CTTTCAGGTT ACTTGGTCGA AACTATCGTA CGTAGGAAGC TATCGAGTCG

        250        260        270        280        290        300
TGGTAGAGCG GAGGACTGTA GATTGTATAG ACATTTGCGG ACATCCTTAG GTCGCTGGTT
ACCATCTCGC CTCCTGACAT CTAACATATC TGTAAACGCC TGTAGGAATC CAGCGACCAA

        310        320        330        340        350        360
CGATTCCAGC TCGAAGGAAG TGCGTGATGC TTTTGGTTAA AAGCCCTGCA GCTTCCAAGT
GCTAAGGTCG AGCTTCCTTC ACGCACTACG AAAACCAATT TTCGGGACGT CGAAGGTTCA

        370        380        390        400        410        420
AGTAACCACA CTCTCCCGGG AAAACACCCA CGAAGTCTTT CCTGATCACC TAGCTTCCCA
TCATTGGTGT GAGAGGGCCC TTTTGTGGGT GCTTCAGAAA GGACTAGTGG ATCGAAGGGT
```

(pM612) at positions 273 to 278 (Fig. 17) is a short region of DNA which can also be found in the 5'-flanking region (position 183 to 188; Fig. 17).

The plasmid pJM4 contains a human tRNA^{Tyr} gene (Fig. 18). This gene is also 73-bp in length, and the coding sequence is identical with that of the tRNA^{Tyr} gene in pM6. The striking feature of this gene is a 20-bp intervening sequence, whereas the genes contained within the cluster in λHtM6 have introns of 21-bp. The intervening sequence interrupting the coding sequence of this gene does not show sequence homology with the other human tRNA^{Tyr} gene introns except for the same 3'-terminal GAC sequence. This may suggest that this particular sequence has a functional significance. Although the sequence of this intron (pJM4) is different from the others described above, it also has a short sequence at positions 228 to 234 (Fig. 18) which can be found in the 5'-flanking region at positions 156 to 161. The termination signal for this gene is unusual in that it consists of a tract of 8 'T' residues.

A comparison of a *X. laevis* tRNA^{Tyr} gene (Müller and Clarkson, 1980), a human tRNA^{Tyr} gene isolated by van Tol *et al.* (1987) and the five tRNA^{Tyr} genes from this study is shown in Fig. 19. There is considerable homology between the gene flanking sequences. Although the solitary tRNA^{Tyr} gene in pJM4 has substantially different flanking sequences, there are a number of common structural features. The sequence 5'-TCTTC-3', located at various relative positions, can be found in the 5'-flank of the genes within pJM4, pM6128 and pM612. As well the sequence 5'-CTTTCCT-3' is

Figure 18. Nucleotide sequence of the tRNA^{Tyr} gene within pJM4. The upper strand of DNA depicts the non-coding strand of DNA. The tRNA gene is boxed and starts at position 186. The dots under the sequence indicate a region of homology within the intervening sequence and the 5'-flanking region of this gene. The overlined tract of 'T' residues in the 3'-flanking region is the putative transcription termination site.

```
         10         20         30         40         50         60
CCCGGGTCAG TCAGGCTAGC GAGCCGGAGC GTTCTGTCTT TCTGCGCACG CGTAGAGCAC
GGGCCCAGTC AGTCCGATCG CTCGGCCTCG CAAGACAGAA AGACGCGTGC GCATCTCGTG

         70         80         90        100        110        120
ACAGGCCGGC TCTGGGGCTC TGCGCTCCTC GGATTACGCA TGCTCAGTGC AATCTTCGGT
TGTCCGGCCG AGACCCCGAG ACGCGAGGAG CCTAATGCGT ACGAGTCACG TTAGAAGCCA

        130        140        150        160        170        180
TGCCTGGACT AGCGCTCCGG TTTTTCTGTG CTGAACCTCA GGGGACGCCG ACACACGTAC
ACGGACCTGA TCGCGAGGCC AAAAAGACAC GACTTGGAGT CCCCTGCGGC TGTGTGCATG

        190        200        210        220        230        240
ACGTCCCTTC GATAGCTCAG CTGGTAGAGC GGAGGACTGT AGCTACTTCC TCAGCAGGAG
TGCAGGGAAG CTATCGAGTC GACCATCTCG CCTCCTGACA TCGATGAAGG AGTCGTCCTC

        250        260        270        280        290        300
ACATCCTTAG GTCGCTGGTT CGATTCCGGC TCGAAGGAGA CAAGTGCGGT TTTTTTTCTC
TGTAGGAATC CAGCGACCAA GCTAAGGCCG AGCTTCCTCT GTTCACGCCA AAAAAAAGAG

        310        320        330        340        350        360
CAGCTCCCGA TGACTTATGG CACTTTCCTT GGGTGCCTTC AGTGACACAT TGCATTCCAA
GTCGAGGGCT ACTGAATACC GTGAAAGGAA CCCACGGAAG TCACTGTGTA ACGTAAGGTT

        370        380        390        400        410        420
CGAGCAGTTT GAAAGTCTAG CGCTTTCTCC CCATTTTGGG CCTCCCAGCC TGCACGGTAA
GCTCGTCAAA CTTTCAGATC GCGAAAGAGG GGTAAAACCC GGAGGGTCGG ACGTGCCATT
```

Figure 19. Comparison of the tRNA^{Tyr} gene regions from humans and *X. laevis.* The tRNA genes are shaded. Regions of homology are indicated by boxed areas, $\overline{\text{or}}$ are either underlined or overlined. Sequence (1) is that reported for a tRNA^{Tyr} gene from *X. laevis* (Müller and Clarkson, 1980). Sequence (2) is a human tRNA^{Tyr} gene reported by van Tol *et al.* (1987). Sequence (3) is the gene in pJM4. Sequence (4) is the tRNA^{Tyr} gene carried within pM6. Sequence (5) is the gene within pM6128. Sequence (6) is the nucleotide sequence for the gene contained in pM6IT. Sequence (7) is the human tRNA^{Tyr} gene found within pM612. The arrow heads indicate differences between the sequence reported by van Tol *et al.* (1987) and the human tRNA^{Tyr} gene sequences determined in this study.

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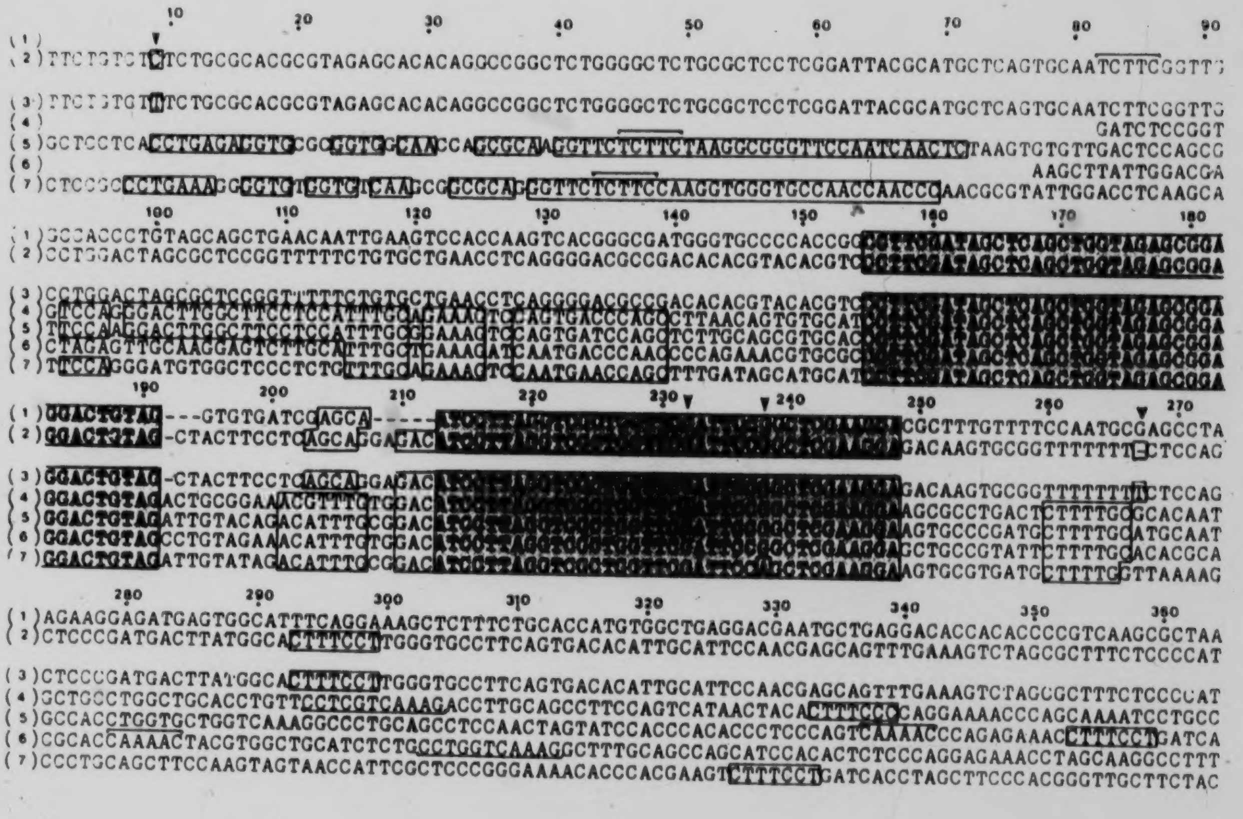


present in all the 3'-flanking regions of the human tRNA^{Tyr} genes, except for the gene within pM6IT. The four tRNA genes of the cluster in λHtM6 have considerable flanking sequence homology. The 5'-flanking region from -16 to -42 is almost identical in these four genes (Fig. 19).

The gene contained within pJM4 is identical with the gene reported by van Tol *et al.* (1987). In fact the recombinant bacteriophage λHtM4 appears to be very similar if not identical to the clone of van Tol *et al* (1987). The probable fortuitous isolation of the same human DNA clone has led to an interesting result. Although the fragment pattern for these two clones appears to be identical, there are two differences in the nucleotide sequences. Figure 19 indicates a T to C transition between the 5'-flanking sequences of these genes. The tRNA precursor encoded within pJM4 is terminated by a tract of 8 'T' residues, but the gene reported by van Tol *et al.* (1987) would use a sequence of 7 'T' residues (Fig. 20). Figure 21 shows the isolated tRNA^{Tyr}'s drawn in the familiar cloverleaf structure.

The products of *in vitro* transcription of the human tRNA^{Tyr} genes by HeLa cell extracts prepared as described by Manley *et al.* (1980) were analysed by PAGE and radioautography. The radioautograph of the RNA products (Fig. 22) demonstrates the varying rates of transcription and varying degrees of processing observed with the different human tRNA^{Tyr} genes.

The tRNA^{Tyr} genes examined all showed synthesis of putative tRNA precursors, although the rates of expression varied greatly. The tRNA gene within the plasmid pM6 showed the lowest rate of

Figure 20. Radioautographs of DNA sequence gels. (A) A region of sequence is shown (249 to 273; Fig 23) which illustrates the tract of eight 'T's which terminate the $tRNA^{Tyr}$ gene contained within the recombinant plasmid pJM4. (B) Sequence spanning a region of DNA (3 to 14; Fig 19) which is identical to DNA sequence reported by van Tol *et al.* (1987) except for the indicated T residue. (C) Sequence indicating the base change at position 63 in the $tRNA^{Tyr}$ gene (225 to 250; Fig 19) within the recombinant plasmid pM612.

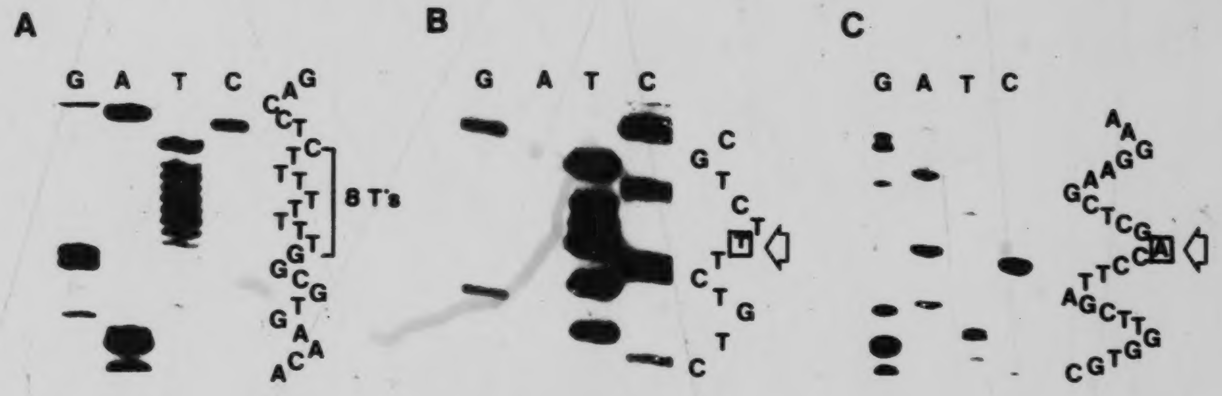
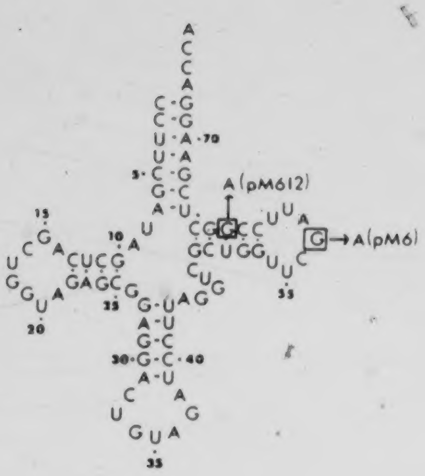

A
G A T C
CAGCCTCTTTTTTTTGGCGGTGAAACA
8 T's
B
G A T C
CGTCTTCTTGTC
C
G A T C
AAGGAAGCTCGACCTTAGCTTGGTGC

Figure 21. Potential secondary structure of five human tRNA^{Tyr}s and of the anticodon regions of their primary transcripts. For completeness the top figure has the 3'-CCA terminus which is not encoded in the DNA sequence. The bases are shown in their unmodified forms. The boxed nucleotides indicate base changes between the tRNA^{Tyr} genes and indicate in which genes the polymorphisms occur. The lower figures depict possible secondary structures which the intervening sequences in the anticodon stem and loop could assume. The underlined 5'-GUA-3' sequences are the anticodons of the tyrosine tRNAs. The arrows indicate where the pre-tRNA^{Tyr}s are spliced to produce the mature tRNAs. The structure drawn for the tRNA^{Tyr} contained within pJM4, with an extended anticodon stem-loop, is unlikely to form *in vitro* or *in vivo* because it is not likely to be stable.

2


A(pM612)
G→A(pM6)

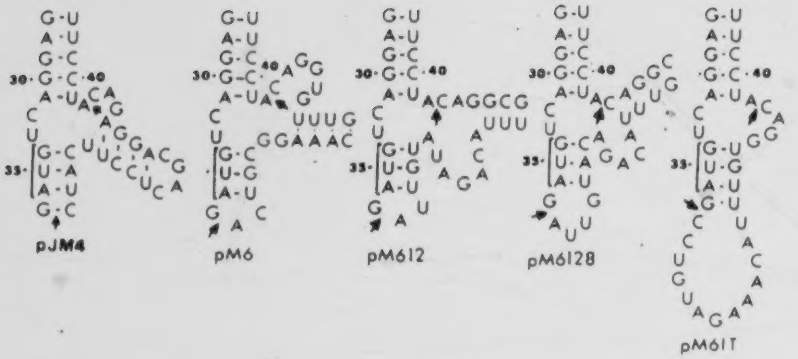
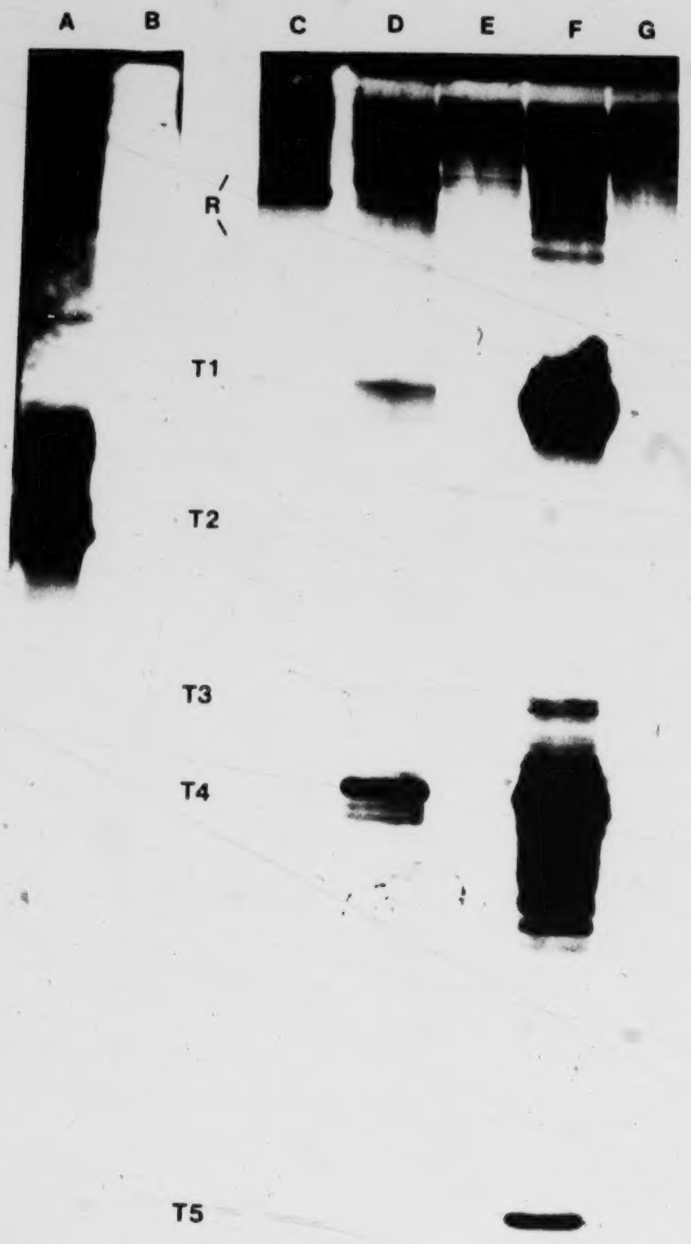

pJM4
pM6
pM612
pM6128
pM61T
30
35
40

Figure 22. Radioautograph of *in vitro* transcription products directed by five human tRNA^{Tyr} genes. One pmole of plasmid DNA was used in each reaction, and the RNA products were separated on a 12% polyacrylamide, 8.3 M urea gel. Lane A is a positive transcription control using a human tRNA^{Lys} gene, where the lower band is the mature tRNA (Roy *et al.*, 1982). The RNA products of the genes contained within the plasmids pAT153, pJM4, pM6128, pM6, pM6IT and pM612 are shown in lanes B through G. As a control, pAT153 was tested to determine if it could direct *in vitro* transcription. As shown in lane B, no RNA products were transcribed from pAT153. The RNA products have been designated with transcription (T) numbers, where T1 is presumed to be a pre-tRNA sized transcript. T2 is a mature sized tRNA product (not visiable in this photograph because it was very faint in the original radioautograph) since it runs with the same mobility as an authentic human tRNA (lysine). T3 is presumed to be a pre-tRNA sized molecule which has been cut at one splice site and therefore contains RNA corresponding to the intron. T4 is presumed to include both half-sized tRNA molecules, derived from complete removal of the RNA which corresponds to the intron. T5 is likely to be the intron. The large RNA transcripts denoted by R are assumed to be long unterminated transcripts. The lighter bands are likely contaminating exonuclease breakdown products of the precursor tRNA molecules.


A
B
C
D
E
F
G
R
T1
T2
T3
T4
T5

transcription whereas the gene contained in pM612 directed the greatest amount of synthesis of tRNA precursor. The gene in pJM4 directed a lower amount of precursor. The genes within pM6128 and pM6IT were expressed with lower but similar efficiencies.

Of the five $tRNA^{Tyr}$ genes isolated, only the gene contained within pJM4 appeared to direct the synthesis of any mature tRNA-sized products. The remaining genes, those within λHtM6, direct the synthesis only of tRNA precursor-sized molecules. These appear to be cleaved at least once (at either the 5'- or 3'-splice site). Although the splicing reaction appears to be inefficient, the liberated intron is observed indicating that the lack of mature tRNA-sized molecules may be the result of diminished splicing endonuclease activity. Alternatively, the ligase activity of the HeLa cell extract may have been low. Since the gene-coding sequences appear to contain all of the nucleotides essential for a functional tRNA gene (Sharp *et al.*, 1985) it is likely that these human $tRNA^{Tyr}$ genes are not pseudogenes.

DISCUSSION

The limited information on complete families of human tRNA genes prompted a search for several members of a single tRNA gene family. The primary aim of this project was to collate several tRNA genes, which accept the same amino acid, with respect to their genomic organization by physical mapping of the segments of human DNA within which they occur. The nucleotide sequences were determined to ascertain if the tRNA genes had identical nucleotide sequences. An additional objective was to determine if variations in the tRNA gene sequence could affect gene expression in a homologous *in vitro* transcription system.

As precedents exist for the occurrence of intervening sequences in other eukaryotic $tRNA^{Tyr}$ genes, as in yeast (Goodman *et al.,* 1977) and *X. laevis* (Müller and Clarkson, 1980), it was suspected that other eukaryotic $tRNA^{Tyr}$ genes would also be interrupted by introns. This expectation has recently been born out with *D. melanogaster* $tRNA^{Tyr}$ genes (Kubli *et al.,* 1988). Thus, a secondary aim of this project was to determine if advanced eukaryotes such as humans would also have intervening sequences, and, if so, determine the extent of homology between these introns.

The cloning of a specific protein-encoding gene is often accomplished by using the mRNA complement of that gene (McReynolds *et al.*, 1977) or by the construction of a single stranded oligonucleotide which is complementary to a portion of the gene sequence (van Tol *et al.*, 1987). With tRNA genes, however, the product of the gene is an RNA molecule of small size, usually less than 80 nt. The tRNAs' small size, extensive secondary and tertiary structure, and frequent contamination with the breakdown products of rRNAs, makes the use of tRNA as a probe extremely difficult (Roy, K. L.; Pirtle, R. M., personal communication). A DNA probe would be preferable since it can be easily purified, as in the large scale purification of an ovalbumin gene by reverse phase column chromatography (Woo *et al.*, 1978). A probe can also be constructed by subcloning a desired fragment containing a homologous gene from another organism to a plasmid vector. Because a specific tRNA^{Tyr} gene probe was available (Lam, W. and Roy, K. L., unpublished), a human-λ recombinant DNA library was screened for tRNA^{Tyr} genes using the *in situ* procedure of Benton and Davis (1977).

Three recombinant clones were isolated which contained human DNA inserts of 16-kb, 16.7-kb and 15.5-kb, and which all encoded tRNA^{Tyr}s. Two of these recombinant DNAs (λHtM2 and λHtM4) had one tRNA^{Tyr} gene each while λHtM6 contained a cluster of at least four tRNA^{Tyr} genes within a 9.2-kb length of DNA. Transfer RNA gene clusters have been previously shown to be present in higher eukaryotic organisms. Roy *et al.* (1982) analyzed a human tRNA gene cluster with tRNA^{Leu}, tRNA^{Lys} and tRNA^{Gln} genes within 1.6-kb of DNA. Doran *et al.* (1987) characterized a human tRNA gene cluster with two tRNA^{Lys} genes and the first reported tRNA^{Phe} genes within 3.7-kb of DNA. A third human tRNA gene cluster has proline and threonine tRNA genes within 724-bp of each other, while a proline-leucine tRNA gene pair is found within 3-kb of the first cluster (Chang *et al.*, 1986). Arrangement of tRNA genes into clusters has also been described for other vertebrate genomes.

At least three clusters have been found in rats (Makowski *et al.*, 1983; Rosen *et al.*, 1984; Sekiya *et al.*, 1982). A cluster of eight tRNA genes (Müller *et al.*, 1987) which is tandemly repeated at least 100 times on a single chromosome in *X. laevis* has been reported by Fostel *et al.* (1984). Although tRNA gene clusters have been described previously for several vertebrate organisms λHtM6 is the first case in which the tRNA genes have apparently encoded the same tRNA species.

There appear to be at least three organizational arrangements for eukaryotic tRNA genes; the highly dispersed single tRNA genes, semi-dispersed clusters where small groups of closely linked tRNA genes are located within a limited distance of each other, and larger, tightly linked clusters containing three or more genes.

Gouilloud and Clarkson (1986) reported a solitary $tRNA^{Tyr}$ gene identical in sequence to that found within the *X. laevis* tDNA gene cluster. There are several other examples of solitary tRNA genes in the human genome (Santos and Zasloff, 1981; Arnold *et al.*, 1986; Goddard *et al.*, 1983; Shortridge *et al.*, 1985).

Ma *et al.* (1984), in their study of two solitary human $tRNA^{Asn}$ genes, reported the first examples of highly conserved flanking sequences. Less highly conserved flanking homologies have been described by Santos and Zasloff (1981) and MacPherson and Roy (1986). The former authors studied two unlinked $tRNA_i^{Met}$ genes while the more recent study focused on two closely-linked $tRNA^{Tyr}$ genes, those of pM6 and pM6128.

A surprising finding is the report by van Tol *et al.* (1987) of another human $tRNA^{Tyr}$ gene (λHtT1) which is embedded in almost identical flanking sequence to that of λHtM4. There is no example of two solitary tRNA genes (of any family) with such an extensive conservation of sequence. There are, however, two changes in the flanking sequence reported in this study (λHtM4) from that reported for λHtT1. A C/T polymorphism exists 145 nt upstream from the 5'-end of these genes. The termination signal for the $tRNA^{Tyr}$ genes encoded by λHtM4 consists of a tract of eight 'T' residues whereas the $tRNA^{Tyr}$ gene described by van Tol *et al.* (1987) is terminated by a tract of seven 'T' residues. Since both human $tRNA^{Tyr}$ genes were isolated from the same human placental DNA library it is possible that these differences are minor polymorphisms between maternal and paternal DNA sequence. It should be pointed out that the sequence polymorphism at -145 could also be the result of errors in reading the DNA sequence. This is unlikely to be the correct explanation for the difference between seven and eight 'T' residues found in the 3'-regions as such runs of single nucleotides are rarely mis-interpreted.

There is considerable shared flanking sequence homology between the five human $tRNA^{Tyr}$ genes sequenced in this study. The sequence 5'-TCTT-3' can be found in the 3'-flank of the $tRNA^{Tyr}$ genes encoded within pM6, pM6IT and pM612. This same sequence can also be found as part of the termination signal for a $tRNA^{Gly}$ (Shortridge *et al.*, 1985), a $tRNA^{Lys}$ and $tRNA^{Gln}$ (Roy *et al.*, 1982) and a $tRNA^{Lys}$ gene described by Doran *et al.* (1987). Consistent with this is the observation that the sequence 5'-GTTT-3' can be found within the termination sequence for three human $tRNA^{Tyr}$ genes (this report and van Tol *et al.*, 1987), an *X. laevis* $tRNA^{Tyr}$ gene

(Müller and Clarkson, 1980), and a human $tRNA^{Phe}$ and $tRNA^{Lys}$ genes (Doran *et al.,* 1987). This sequence can also be found within the termination sequence of a $tRNA^{Pro}$, $tRNA^{Glu}$, and $tRNA^{Leu}$ genes (Chang *et al.,* 1986; Goddard *et al.,* 1983; McLaren and Goddard, 1986). A similar sequence can be found in the 3'-flanking region of several tRNA genes. 5'-TTTCCC-3' can be found in the 3'-flank of a plant $tRNA^{Tyr}$ gene (van Tol *et al.,* 1987), and a human $tRNA^{Phe}$ gene (Doran *et al.,* 1987). The related sequence 5'-TTTCCT-3' can be found in the 3'-flanking region of the human $tRNA^{Tyr}$ genes encoded within pJM4, pM6128 and pM612. It is also present in the 3'-flanking region of a human $tRNA^{Val}$ gene (Arnold *et al.,* 1986) and a human $tRNA^{Phe}$ gene (Doran *et al.,* 1987). These conserved sequences may be the remnants of sequences from a common ancestral tRNA gene. It is also possible that these sequences have an effect on transcription in unknown ways.

There do not appear to be common sequences within the 5'-flanking sequences of various tRNA genes from different organisms. There is, however, considerable patchwork homology between the four human $tRNA^{Tyr}$ genes clustered together within λHtM6. The sequence 5'-YRTTTGCNGAAAGNYCARTGANCCARC-3' can be found in front of each human $tRNA^{Tyr}$ gene within the cluster at the same location. There are also other smaller regions of homology. The functional significance of these regions is not known, although it has been suggested that upstream regions may have an effect on the levels of tRNA gene transcription. The variable levels of *in vitro* transcription observed with the human $tRNA^{Tyr}$ genes in this study are likely a consequence of the different 5'-flanking sequences of these tRNA^{Tyr} genes.

All five human tRNA^{Tyr} genes described in this study are 93 or 94 nt in length, and have considerable homology with the *X. laevis* tRNA^{Tyr} gene. The coding regions for the genes within pJM4, pM6128 and pM6IT are identical to the *X. laevis* tRNA^{Tyr} gene. The tRNA^{Tyr} gene within pM6 has a single polymorphism at position 57 of the mature tRNA^{Tyr}. The tRNA gene located within pM612 has a unique base change at position 63 of the mature tRNA. A polymorphism at this position has not been previously reported in any higher eukaryotic tRNA^{Tyr} gene (Sprinzl *et al.,* 1987). A predicted feature of these genes is the presence of 20- or 21-bp intervening sequences located one bp 3' to the anticodon, in contrast to the *X. laevis* tRNA^{Tyr} gene intron, which begins immediately adjacent to the anticodon on the 3'-side. Although there is almost complete conservation of both the *X. laevis* and human tRNA^{Tyr} gene exons, there is a complete lack of homology between the intervening sequences. The introns within the human tRNA^{Tyr} genes appear to have had very little evolutionary constraint to maintain a particular sequence. The gene found in pJM4 has an intervening sequence of 20-bp. Those genes found within the cluster contained in λHtM6 have introns of 21-bp and have little homology with the other tRNA^{Tyr} gene intron (λHtM4). The intervening sequences in pM6 and pM6IT are conserved in 17 of the 21 nt. Twenty of the 21-bp are homologous between the gene introns encoded by pM6128 and pM612. However, there are only eight common nt between these four intervening sequences. The only common sequence with all six human tRNA^{Tyr}

gene introns is the 3'-terminal GAC. The sequence 5'-AGCA-3' can be found within the intron of the *X. laevis* $tRNA^{Tyr}$ gene and the solitary human $tRNA^{Tyr}$ gene (λHtM4). It is not surprising that the introns between the clustered $tRNA^{Tyr}$ genes and the single gene show little sequence homology since a solitary *X. laevis* $tRNA^{Tyr}$ gene also has a shorter intron and little homology with the clustered *X. laevis* $tRNA^{Tyr}$ gene (Gouilloud and Clarkson, 1986). This is in contrast to the observation of yeast $tRNA^{Tyr}$ gene introns. Eight solitary $tRNA^{Tyr}$ genes exist in the genome of *S. cerevisiae* on six different chromosomes, and all these genes are interrupted by identical introns (Olson *et al.*, 1977). Kubli *et al.* (1988) have reported *Drosophila* $tRNA^{Tyr}$ genes which also are interrupted by intervening sequences of variable length and have different sequence. These genes have three size classes of introns: 20/21-bp, 48-bp and 113-bp. It is interesting to speculate that a further search of the human genome may yield $tRNA^{Tyr}$ genes with introns longer than 21-bp since *Drosophila* has three size classes of introns.

S. cerevisiae contains nine families of tRNA genes which are interrupted by introns (Ogden *et al.*, 1984). *D. melanogaster* appears to have only two families of tRNA genes which contain introns, those for leucine and tyrosine tRNAs. The only reported intron within a tRNA gene in *X. laevis* occurs in a $tRNA^{Tyr}$ gene. It is possible that the introns occur only within the $tRNA^{Tyr}$ genes in humans, since more advanced eukaryotic organisms seem to follow a general pattern of the loss of introns within their tRNA genes.

van Tol and Beier (1988) have suggested that all human tRNA^{Tyr} genes contain introns on the basis of hybridization evidence. Only mature tRNA^{Tyr}s have a pseudouridine modification in the centre of the anticodon, presumably to maintain codon efficiency. It has been shown, in two separate studies with different organisms, that the presence of the intron is essential for this modification to occur (Johnson and Abelson, 1983; van Tol and Beier, 1988). The enzyme RNase P is responsible for the 5'-end maturation of the pre-tRNA. The intron within a yeast tRNA^{Leu} gene is important for this 5'-end processing by RNase P (Leontis *et al.*, 1988). It is possible that humans maintained the intron within their tRNA^{Tyr} gene family to ensure the existence of the pseudouridine modification in the centre of their anticodon. It is also possible that humans have maintained the intron within the tRNA^{Tyr} genes for other, as yet, unknown reasons.

The nucleotide sequence of a bovine liver tRNA^{Tyr} and a *D. melanogaster* tRNA^{Tyr} have been reported (Johnson *et al.*, 1985; Suter *et al.*, 1986). Those genes within the plasmids pJM4, pM6128 and pM6IT would be expected to direct the synthesis of a tRNA with almost identical primary sequence to the bovine tRNA^{Tyr}. A single change would occur at position 16 where the human tRNA^{Tyr}s would contain a C, whereas the bovine tRNA^{Tyr} would have a D. One *D. melanogaster* tyrosine tRNA, however, has three base changes which would not be expected to occur in the human tRNAs expressed from the genes isolated in this study; a Ψ28, A42 and an A59 in the mature tRNA molecule. van Tol *et al.* (1987) also reported the nucleotide sequence of a human tRNA^{Tyr} which could be synthesized from the genes encoded within pJM4, pM6128 and pM6IT. The genes contained in pM612 and pM6 are expected to direct the synthesis of $tRNA^{Tyr}s$ which would have polymorphisms at positions 63 (G/A) and 57 (G/A), respectively. The $tRNA^{Tyr}$ genes in pM6 and pM612 are presumed to be minor species of $tRNA^{Tyr}$ in human liver and placenta as these species have not been described by other workers (Johnson *et al.,* 1985; van Tol *et al.,* 1987). There is one $tRNA^{Tyr}$ from *D. melanogaster* (Suter *et al.*, 1986) which does not have a queuosine modification in the first position of the anticodon. However, the fact that all other sequenced $tRNA^{Tyr}s$ do have the queuosine modification would suggest that the human $tRNA^{Tyr}$ species encoded by the genes reported in this study also contain this hypermodified base in the mature tRNA.

Variable efficiencies of transcription from identical tRNA genes have been observed by various researchers (Doran *et al.*, 1987; Arnold *et al.*, 1986; see also Sharp *et al.*, 1985). It has been shown in several cases that the 5'-flanking sequence has a direct effect on the level of tRNA gene transcription (Sajjadi *et al.*, 1987; Cooley *et al.*, 1984; Sprague *et al.*, 1980). It is therefore not surprizing, since each $tRNA^{Tyr}$ gene has a different 5'-flanking sequence, that these genes are apparently expressed at varied levels in an *in vitro* transcription system.

Since transcription-modulating elements for tRNA genes are not evident from the nucleotide sequence (Sharp *et al.*, 1985), it is difficult to determine which part of the 5'-flanking sequence has an effect on gene transcription. It is unlikely that one unique sequence enhances the level of expression of all tRNA genes. Recently, two different upstream sequences have been suggested to enhance the expression of a *D. melanogaster* 5S RNA gene and a U6 RNA gene (Garcia *et al.*, 1987; Bark *et al.*, 1987). These genes are also transcribed by RNA polymerase III. The sequence 5'-GAGTATAA-3', determined to aid in the expression of a *D. melanogaster* 5S RNA gene, is not present in the nucleotide sequence upstream of the human tRNA genes. Bark *et al.* (1987) have suggested that the sequence 5'-ATTTGCAT-3' located upstream from a U6 RNA gene enhances its' expression. A similar sequence, 5'-GTTTGCAG-3', is located 34-bp upstream from the transcription start site of the tRNA gene encoded within pM612. Interestingly, this gene is expressed at the highest level in the *in vitro* transcription experiments. However, the similar sequence 5'-ATTTGCAG-3' is also positioned 34-bp upstream from the start site of the tRNA gene contained within pM6, the gene which had the lowest transcription efficiency. Although two different motifs have been presented for the transcription of two classes of RNA polymerase III dependent genes, it is not known whether tRNA genes also have a requirement for the presence of a unique sequence which increases expression.

There is no direct evidence to suggest that any of these genes have arisen by gene duplication, although the extensive similarities between the flanking sequences do give some credibility to this theory. The fact that all six genes showed at least minimal expression in an homologous *in vitro* transcription system strongly indicates that these genes would be active *in vivo*.

The isolation of six human tyrosine-accepting tRNA genes should prove useful in determining the importance of flanking sequence influence on the rate of expression of tRNA genes. Since these genes are embedded in different flanking sequence and are transcribed with different levels of efficiency it may be possible to determine (at least for one tRNA gene family) those sequences which are responsible for modulation of tRNA gene expression.

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GLOSSARY

Amplification: refers to the synthesis of additional copies of a sequence of DNA.

Anticodon: is a sequence of three nucleotides in a constant position in the structure of tRNA that is complementary to the codon(s) in mRNA to which the tRNA binds.

Cluster: a region of DNA that contains two or more tRNA genes that can be isolated on a single bacteriophage DNA molecule.

Codon: is a sequence of three nucleotides in mRNA that specifies an amino acid or a termination signal in protein biosynthesis.

Colony hybridization: is a technique in which DNA from a single bacterial colony is immobilized on a membrane in order to identify bacteria carrying recombinant plasmids whose inserted DNA is homologous with some particular DNA sequence.

Consensus sequence: is an idealized sequence in which each position represents the base most often found when several actual sequences are compared.

Divergence: is the percent difference in nucleotide sequence between two related DNA sequences.

DNA library: is a collection of cloned DNA fragments that together represent a substantial proportion of the complete genome of an organism.

Downstream: refers to sequences which occur farther in the direction of transcription of the gene.

End-labeling: is a procedure which allows the addition of a radioactively labeled nucleotide or phosphate to one end (5' or 3') of a DNA or RNA strand.

Enhancer element: is a sequence of DNA that increases the utilization of (some) eukaryotic promoters in *cis* configuration, but can function in any location, upstream or downstream relative to the promoter.

Exon: is any segment of an interrupted gene that is represented in the mature RNA product.

Flanking regions: are the DNA sequence before and after a gene coding region.

Hybridization: is the pairing of complementary RNA or DNA to a sequence of DNA.

Intervening sequence: a segment of DNA that is transcribed, but is removed from within the transcript by splicing together the sequences (exons) on either side of it.

Introns: are intervening sequences.

Isoaccepting tRNA: tRNA molecules which have different anticodons but accept the same amino acid.

Leader: sequence is the 5'-RNA sequence which precedes the mature 5'-end of the tRNA.

Loop: is a single stranded region at the end of a stem in RNA which corresponds to the region of RNA between the inverted repeats which form the stem.

Modification: refers to any change made to a nucleotide after it has been incorporated into a polynucleotide chain.

Nick: in duplex DNA describes the absence of a phosphodiester bond between two adjacent nucleotides on only one strand of the duplex.

Nick-translation: describes the ability of *E. coli* DNA polymerase I to use a nick as a starting point from which one strand of duplex DNA is degraded and replaced by resynthesis of new DNA; this is used to introduce radioactively labeled nucleotides into DNA.

Non-coding strand: of DNA has the same sequence as its' RNA product.

Oligonucleotide: a short single-stranded DNA or RNA molecule, usually less than 20 nucleotides in length.

Patchwork homology: refers to short regions of DNA which are similar in sequence with DNA from another region of the genome or from another organism, separated by dissimilar sequences.

Polymorphism: refers to the existence, within a population, of a change in the amino acid sequence of a polypeptide that cannot be attributed to recurrent mutation alone. It also refers to the occurrence in a family of tRNA genes of single base changes in the DNA sequence.

Pre-tRNA: is the original unmodified RNA transcript corresponding to the complete transcription unit including some flanking sequence.

Pseudogenes: are inactive but stable components of the genome derived by mutation of an ancestral active gene, or by reverse transcription of a processed RNA molecule.

Southern transfer: is the procedure for transferring denatured DNA from a separating gel (either agarose or polyacrylamide) to a membrane filter so that the DNA bound to the filter can be hybridized to a complementary nucleic acid.

Splicing: describes the removal of introns and joining of exons in RNA: thus introns are spliced out, while exons are spliced together.

Stem: describes a double-stranded region formed by base pairing between adjacent (inverted) complementary sequences in a single strand of DNA or RNA.

Structural gene: codes for any RNA product or protein.

Suppressor: is usually a gene encoding a altered tRNA that reads a mutant codon, either in the sense of the original codon or to give an acceptable substitute.

Thalassemia: is a disease of red blood cells resulting from the absence of either α or β globin.

Transfection: is transformation of infectious DNA.

Transformation: is a genetic change effected in a cell as the result of the incorporation of DNA from a virus or some genetically different cell type; also refers to the taking up of extraneous genetic material by salt-treated bacterial cells in genetic manipulation.

Transition: is a mutation in which one pyrimidine is substituted by the other or in which one purine is substituted by the other.

tRNA gene family: refers to a population of tRNA molecules which accept the same amino acid.

Upstream: refers to sequences proceeding in the opposite direction from transcription.

U RNAs: are highly abundant small nuclear RNAs involved in the processing of mRNA.